THE PRIMARY STRUCTURE OF LEUCINE AMINOPEPTIDASE

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LEUCINE AMINOPEPTIDASE FROM BOVINE EYE LENS

PROPERTIES, STRUCTURE AND A COMPARISON WITH PORCINE KIDNEY LEUCINE AMINOPEPTIDASE FROM CYTOSOL

Leucine aminopeptidase (α-aminoacylpeptide hydrolase (cytosol), EC 3.4.11.1) from bovine lens tissue was purified in crystalline form by the group of Hanson in 1965[1]. The enzyme had many properties in common with leucine aminopeptidase (LAP)[1] isolated from porcine kidney cytosol, the first enzyme of this class of metallopeptidase to be studied in detail, by Smith and Spackman[2]. Later on the lens enzyme appeared to be almost identical to LAP purified from pig kidney[3,4,5]. The original name leucine aminopeptidase was attached to the enzyme, because L-leucinamide, in addition to leucine peptides, was hydrolyzed, whereas *N*-acylated substrates were resistant. This name has persisted, although it is now clearly recognized that hydrolysis is not restricted to leucyl compounds[6]. The recommended systematical name is now α-aminoacylpeptide hydrolase (cytosol).

The best studied *N*-terminal metallo-exopeptidase at this moment is LAP from bovine lens, probably because this tissue contains the enzyme in abundance and the purification from bovine lens is rapid and simple. Aminopeptidases (EC 3.4.11.1) of broad specificity have been isolated and purified from several animal organs and have been studied to varying degrees. However, identification has been inadequate, because the enzymes have not completely been purified, and since most tissues contain other proteases, whose specificities overlap with that of the aminopeptidases, conclusive comparison is not possible.

As already stated, bovine lens LAP is almost identical to the enzyme purified from pig kidney. Both enzymes have the same molecular weight, the same subunit molecular weight, and almost identical amino acid compositions[4]. Comparison of the two enzymes on basis of microcomplement fixation indicated an amino acid sequence difference of 8%[5]. An enzyme with comparable molecular properties has also been isolated from porcine liver[7].

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[1] The abbreviations used are: LAP, leucine aminopeptidase; M_r, molecular weight.

A review on bovine lens leucine aminopeptidase has been published by Hanson and Frohne[8]. LAP hydrolyzes the peptide bond of all *N*-terminal L-amino acids except proline and hydroxyproline. It does not hydrolyze the β- and γ-bonds of aspartic acid and glutamic acid, respectively. Furthermore, it hydrolyzes amino acid amides, alkylamides, arylamides and hydrazydes. The *N*-terminal residue must have the L-configuration, and has to possess a free α-amino group in the unprotonated form. The enzyme removes hydrophobic and aliphatic residues most rapidly, whereas some other residues are removed very slowly[3]. The *exo*peptidase activity has a broad pH optimum between 8.5 and 10.0, depending upon substrate and activating metal ions[3,9].

A molecular weight of 326,000 ± 20,000 for the native LAP molecule has been determined[10,11]. Considering this large size, it was expected to be composed of subunits. In the older literature Hanson's group claimed, that LAP was composed of ten subunits of molecular weight 32,600[12,13,14]. These results were rejected by the group of Carpenter[11,15]. These investigators determined an apparent subunit molecular weight of 54,000 for LAP, on basis of sedimentation equilibrium studies in a variety of denaturing solvents, by gel electrophoresis in sodium dodecyl sulfate, and by cross-linking with dimethylsuberimidate followed by electrophoresis. Our results confirmed the latter value[16].

Cross-linking experiments suggest that LAP is composed of six identical protomers, arranged as a trimer of dimers[15]. Several proposals for the quaternary structure have been made on basis of the structure consisting of six identical subunits. Small angle x-ray scattering studies in solution were in agreement with six subunits arranged in octahedral array[17]. From electron microscope data a model of LAP could be described in the form of a twisted trigonal prism or deformed octahedron, assuming the presence of idealized spherical subunits[18]. Electron microscopy of single molecules and of both stained and unstained thin sections of crystals could be interpreted as a quaternary structure composed of six asymmetric bilobal subunits, arranged so that the principal lobes are eclipsed and the minor lobes are staggered in a manner similar to that of aspartate carbamoyltransferase[19,20,21]. Diffraction data of LAP revealed that the enzyme crystallizes in the hexagonal space group $P6_322$ with unit cell dimensions a=b=132 Å and c=122 Å. Two hexameric molecules are contained in each unit cell. The asymmetric unit consists of one monomer of molecular weight 54,000[22].

The possible bilobal structure of LAP as revealed by electron microscopy

was further studied by means of 'limited proteolysis'. We investigated the possibility to separate the lobes by cleavage of the peptide chain, or to nick the peptide chain in regions of the molecule, which are organized as compact domains. Among the tested enzymes (trypsin, chymotrypsin, plasmin and thrombin) only trypsin was able to produce a cleavage in native LAP, resulting after denaturation in two fragments of M_r 17,000 and 37,000[16,24]. In spite of complete splitting of the bond between arginine-137 and lysine-138, the enzyme retained all of its catalytic properties, due to the fact that the LAP aggregate remained intact. Consequently, the surface topology around the susceptible bond is identical in all subunits[23,24]. It is possible, that the arginine-137 lysine-138 bond is located in a connecting region of the bilobal structure, as proposed by Taylor[20].

LAP from bovine lens is a zinc metallopeptidase. At first Kettmann and Hanson[25] determined a zinc content of 5.0-7.6 g atom per M_r 3.2×10^5. More accurate measurements by the group of Carpenter[26] revealed 12 g atoms of zinc per M_r 324,000 (*i.e.* two zinc ions per subunit). Thus, the native bovine lens enzyme can be represented as follows: $[(LAP)Zn_6Zn_6]$. The enzyme is activated by incubation with either Mg^{2+} or Mn^{2+}. Carpenter *et al*[26,27] have demonstrated that this is due to the replacement by these metals of one Zn^{2+} per subunit, located at the activation site. This activation with Mg^{2+} or Mn^{2+} yields $[(LAP)Zn_6Mg_6]$ and $[(LAP)Zn_6Mn_6]$. Removal of the Zn^{2+} at the other site (referred to as the structural site[27]) results in a total loss of activity, which is regained by reconstitution with Zn^{2+}, but not with Mg^{2+} or Mn^{2+} [26,27].

The splitting of the bond between arginine-137 and lysine-138 by limited tryptic digestion on native LAP caused an acceleration of the activation of LAP by manganese ions. A possible explanation is that the splitting of this bond by a subtle conformational change facilitates the exchange of a zinc ion in the activation site by manganese[16,24].

Accurate determination of the metal binding stoichiometry and of metal ion modulation of cytosol porcine kidney LAP, revealed 6 g atoms of zinc per M_r 320,000 (*i.e.* native form, designated $[(LAP)Zn_6\text{--}]$). The enzyme activated with Mg^{2+} or Mn^{2+} retained the same Zn^{2+} content as before activation. In addition they acquire approximately 6 mol of activating metal ion per hexamer. The Mn^{2+}- and Mg^{2+}-activated hog kidney leucine aminopeptidases from cytosol are, therefore, designated as $[(LAP)Zn_6Mn_6]$ and $[(LAP)Zn_6Mg_6]$, respectively[28]. The nature of the ion occupying the activation site in hog kidney and bovine lens enzyme has a pronounced effect on the maximum velocity

of the reaction and a minor effect on the Michaelis constant[26,28]. The determination of the internuclear distances between Mn^{2+} at the activation site in bovine lens $[(LAP)Zn_6Mn_6]$ and the protons of the enzyme-bound competitive inhibitor *N*-(leucyl)-o-aminobenzenesulfonate revealed that Mn^{2+} is in close proximity to the carbonyl oxygen of peptide substrates, which are bound at the active site of LAP. The activation site can thus be assumed to be part of the active site[29].

It was also possible to replace both Zn^{2+} ions by Co^{2+} ions. Prolonged incubation of bovine lens $[(LAP)Zn_6Zn_6]$ in the presence of $CoCl_2$ yields an active enzyme in which 2 g atoms of Co^{2+} per subunit have replaced the Zn^{2+} (*i.e.* designated $[(LAP)Co_6Co_6]$). Selective back replacement of the Co^{2+} ions by Zn^{2+} ions (*i.e.* preparation of $[(LAP)Co_6Zn_6]$ and $[(LAP)Zn_6Co_6]$) and measurement of the specific activities, revealed that the activity was affected only when cobalt was substituted at the activation site, and that cobalt substitution at the structural site had no effect[30].

Native bovine lens LAP is stable in solution for years, if kept in a refrigerator at 4 °C. At 65 °C the enzyme solution is stable up to 5 min. Lyophilization of enzyme solution leads to inactivation[8]. The enzyme is remarkably stable against denaturing agents like urea[11].

Efforts to design an active site-directed affinity label, which is able to mark a nucleophilic site at the active center, as proposed by Bryce and Rabin[31], have failed. The chloromethyl ketones of *L*-phenylalanine and *L*-leucine appeared to be competitive and reversible inhibitors[32,24]. A series of *N*-leucyl-aminobenzenesulfonylfluoride derivatives, which were designated to be an active site-directed affinity label by analogy to similar compounds that inactivated chymotrypsin, even proved to be quite good substrates[33]. Reaction of the exposed sulfhydryl group with iodoacetamide or *p*-chloromercuribenzoate has no effect on enzyme activity[26,34]. Diisopropyl fluorophosphate, a serine protease inhibitor, also has no effect on the activity of LAP[8]. The only amino acid residue which probably participates in the enzyme mechanism is histidine, because modification of histidine residues by diethylpyrocarbonate affects the enzyme activity[35]. The inhibition of cytosol LAP from porcine kidney by amino acid hydroxamates and related compounds suggests a reaction mechanism in which a zinc-bound hydroxide ion participates in concerted proton transfer processes, while the penta-coordination and charge field at the zinc atom remain unchanged[36].

Although several mechanisms for the action of LAP have been proposed[6,24,31,36],

none has been experimentally confirmed. The interpretation of x-ray crystallographic studies, resulting in a proposal for the tertiary and quaternary structure, probably will provide better understanding of the mechanism of action on a molecular basis.

PROPERTIES OF SOME OTHER MAMMALIAN AMINOPEPTIDASES

Enzymes with aminopeptidase activity occur ubiquitous in nature. They actually form a very heterogeneous group of enzymes, differing in molecular weight, subunit composition, metal content, specificity and kinetic properties. In fact, no detectable structural relationship exists between lens and porcine kidney LAP on the one hand and the other aminopeptidases on the other hand.

Often the term 'leucine aminopeptidase' is also used in clinical practice in reference to human serum aminopeptidase activity, because routine assays usually are performed with L-leucinamide or chromogenic substrates such as L-leucine-β-naphthylamide. This activity actually is a group of several nonspecific aminopeptidases which are chromatographically separable. At least, five closely related alanine aminopeptidases of this 'leucine aminopeptidase' type are distinguishable in human blood on basis of their electrophoretic or chromatographic behavour[37]. Alanine aminopeptidase hydrolyzes maximally when alanine is the *N*-terminal amino acid residue of peptides, and likewise alanine amides and certain chromogenic alanine substrates. Other amino acids with nonpolar R-groups, *e.g.* L-leucine, are also hydrolyzed at a significant rate[38]. The tissues of origin of some of these alanine aminopeptidases have been established; one is from liver, one from kidney, another from duodenum, and two are from pancreas[39].

These alanine aminopeptidases are membrane-bound enzymes. Two enzymes of this group have been purified to homogeneity, after solubilizing the activity by autolysis. Substantial evidence is available indicating that alanine aminopeptidase from human liver is identical, or nearly so, to alanine aminopeptidase from human kidney. Both are glycoproteins of $M_r \pm 240{,}000$ and are composed of two subunits of equal molecular weight (118,000). The enzymes contain one zinc ion per subunit and have nearly equivalent amino acid compositions[40,41]. Another molecular form of human liver alanine aminopeptidase, with M_r 170,000, was obtained by bromelain treatment during purification[42]. A membrane-bound alanine aminopeptidase was also purified from human intest-

ine[43].

The mechanism of attachment to the membranes of aminopeptidases has been studied in the pig and rabbit brush borders of intestinal and renal cells. A survey of the microvillar peptidases has been given by Kenney and Booth[44]. Pig intestinal aminopeptidase N (EC 3.4.11.2) is active on *N*-terminal neutral and basic amino acids in neutral peptides and synthetic derivatives, aminopeptidase A (EC 3.4.11.7) on *N*-terminal acidic amino acids in peptides and synthetic derivatives. The intestinal aminopeptidases N and A are transmembrane proteins composed of a hydrophilic, sugar-rich and enzymatically active 'head' protruding from the external surface of the membrane, and of a short, predominantly hydrophobic 'anchor' spanning the lipid core[45,46].

These enzymes can be purified, yielding a detergent- or a protease form, with and without the hydrophobic anchor peptide, respectively[45,47,48]. The hydrophobic anchor sequence is situated in the *N*-terminal part of the polypeptide chain. The molecular weights of the anchor liberated by trypsin during the conversion of the detergent form into the protease form have been estimated to be about 3500-3700 in case of aminopeptidase N[47] and 4500 in case of aminopeptidase A[46]. The detergent form of rabbit intestinal aminopeptidase N looses a peptide of M_r 3800 during conversion to the hydrophilic form[49]. The *N*-terminal sequence (14 residues) of the anchor of the rabbit enzyme has been determined, and comparison with the amino acid composition suggests, that the anchor peptide is composed of a hydrophobic core of about 20 residues, sandwiched between a positively charged *N*-terminal segment of 4 residues and a *C*-terminal hydrophilic segment, probably cut off from the hydrophilic domain of the enzyme during the limited proteolysis leading to the protease form[50].

The hydrophilic part of porcine aminopeptidase N has a molecular weight of 280,000, and has been found to contain three subunits, of M_r 130,000 (α-chain), 97,000 (β-chain) and 49,000 (γ-chain). The carbohydrate content is 23% and two atoms of zinc are bound per molecule. The amphiphilic form displayed similar polypeptide molecular weights, indicating that the conversion from detergent to protease form involves the removal of a small hydrophobic part of the molecule[51]. When the enzyme was purified without exposure to pancreatic proteolytic enzymes, a dimeric form could be isolated, consisting of two subunits α[52]. The β- and γ-subunits are derived from the α-chain, and both detergent α-subunit and detergent β-polypeptide contain an anchor peptide. The purified form occurs in subunit structures which can be described as αβγ

and $\beta_2\gamma_2$[47,48].

The rabbit aminopeptidase N (M_r 200,000-220,000 in the detergent form) was found to be monomeric after solubilization from the membrane[49]. Aminopeptidase A from hog intestinal brush border has a molecular weight of 247,000 and a subunit of M_r 120,000. The detergent and protease form are probably symmetrical dimers and each subunit of the dimer possesses its own anchor[46]. The brush border aminopeptidases are like all other proteins synthesized inside the cell. Therefore, a post-translational intracellular processing must take place ensuring the passage of the external protein domains across the lipid bilayer and their integration at the proper place[53]. Recently, a hypothesis for the processing of rabbit brush border aminopeptidase N has been proposed on basis of the facilitated assembly to the microsomal membrane caused by a positively charged lysine residue near the beginning of the sequence (lysine position 4) just before an uninterrupted stretch of hydrophobic amino acids[50].

The membrane-bound aminopeptidases are a model system for studying the mode of insertion of intrinsic membrane proteins, and belong together with leucine aminopeptidase to the most extensively studied *exo*peptidases.

SOME ASPECTS OF THE PHYSIOLOGICAL ROLE OF LEUCINE AMINOPEPTIDASE IN INTRACELLULAR PROTEIN BREAKDOWN IN MAMMALIAN CELLS

Since LAP probably is involved in the processes of intracellular protein breakdown, some recent findings on intracellular proteolysis in mammalian cells will briefly be discussed[54,55,56]. Intracellular proteolysis serves a number of physiologically important functions. For example, membrane-associated enzymes are important in the processing of secreted proteins[57], while lysosomal cathepsins appear to be responsible for degrading pinocyted and membrane proteins[55,58], and for accelerated degradation of intracellular proteins during starvation[55,59]. In addition, within the cytoplasm of mammalian cells, there is a soluble ATP-dependent proteolytic system, that selectively degrades polypeptides with abnormal structures, such as may result from mutations, biosynthetic errors, or post-synthetic damage[55,60,61].

Most available evidence indicates that also degradation of short-lived cellular proteins is nonlysosomal in nature[55]. The site of degradation of long-lived cellular proteins remains unclear. Cellular components can be sequestered into autophagic vacuoles where they are degraded by lysosomal en-

zymes[62]; proteins can also be broken down by the ATP-requiring proteolytic system present in the cytosol. It is possible, that the same proteins under different conditions are degraded by different pathways[55]. In spite of the importance of the soluble degradation pathway, only recently efforts have been made to isolate and characterize the enzymes, which may be involved. This ATP-dependent nonlysosomal process has been studied most extensively in reticulocytes[55].

In reticulocyte extracts proteins are degraded completely to amino acids. This process shows an optimum at pH 7.8[60] and must involve multiple proteases, since it is inhibited by diisopropyl fluorophosphate[63] (an inhibitor of serine *endo*proteases), o-phenanthroline[60] (a chelating agent that inhibits metallo-enzymes), and bestatin[64] (which binds to the active site of aminopeptidases). A serine protease with *endo*peptidase activity, which is stimulated by ATP, has been found in reticulocytes and other mammalian cells[65]. This enzyme may catalyze the initial cleavage of substrates, and the products of this serine protease are polypeptides ($M_r \geqslant 2000$), which are presumably degraded by soluble proteases sensitive to o-phenanthroline or bestatin. Alkaline proteases in the cytosol of mammalian cells, including erythrocytes, are able to degrade these polypeptides of $M_r \geqslant$ 2000-3500. A high molecular weight metalloendoprotease with these substrate properties has been purified to homogeneity from different mammalian cells. The products of degradation by this protease are proper substrates for aminopeptidases such as leucine aminopeptidase[63].

Recent studies have shown that the substrates for proteolysis (for instance abnormal proteins) in reticulocytes and in Ehrlich ascites cells first become conjugated to ubiquitin, a small M_r 8500, heat-stable polypeptide of universal occurrence. Several molecules of ubiquitin are bound by isopeptide linkages to ε-NH_2 lysine groups of the substrate[55,66]. The ATP-dependent enzyme system that catalyzes this conjugation has been purified from reticulocytes[67,67a]. The ubiquitin-protein conjugates are degraded rapidly with the release of free and reutilizable ubiquitin[55]. Studies of the proteolysis during maturation of reticulocytes into erythrocytes showed that the ATP-dependent proteolytic activity was found to decrease dramatically with reticulocyte maturation as well as during further aging of the erythrocytes. The ATP-dependent proteolysis remained until most of the ribosomes were degraded and the degradation of reticulocyte proteins associated with cell maturation probably was catalyzed by this ATP-dependent system[68,69].

It has also been reported that bestatin causes accumulation of di- and tripeptide intermediates during the breakdown of cellular proteins in mouse liver. The authors argue that autophagy is responsible for all or most of the accumulated intermediates and that, at least, a large fraction of those found in the cytosol escaped from the organelles[62]. Cytosolic *exo*peptidases, including leucine aminopeptidase will be able to digest these di- and tripeptides.

In summary: in most mammalian cells the cytosolic intracellular protein breakdown seems to proceed in three steps:

1. a proteolytic attack by one or more *endo*peptidases, resulting in polypeptides of $M_r \geqslant 2000$
2. hydrolysis of these peptides to di- and tripeptides by another class of *endo*peptidases
3. hydrolysis to free amino acids by *exo*peptidases, in which leucine aminopeptidase is likely to be involved.

The latter system is also capable of hydrolyzing peptides, which escape from organelles.

DATA CONCERNING INTRACELLULAR PROTEOLYSIS IN THE LENS AND A POSSIBLE ROLE OF LEUCINE AMINOPEPTIDASE IN THIS MECHANISM

The mechanisms of intracellular breakdown of proteins in the eye lens are still quite obscure, albeit in recent years some new aspects of proteolysis in the eye lens have been published.

Histological studies have shown the presence of lysosomes in the bovine lens epithelial cells[70]. The outer cortical region also contains some lysosomal structures, but the inner cortex and nucleus are devoid of these organelles[71]. In the bovine lens a low activity of the lysosomal *exo*peptidase dipeptidyl peptidase II (EC 3.4.14.2) has been recognized[72]. The release of lysosomal cathepsins with acid pH optima is not known in the lens. Extracts of lenses from various species seem to lack the predominance of activity at acid pH values seen in most tissues[73].

In vitro studies show a definite autolysis of lens proteins from various species at a pH optimum around neutrality. Both high temperatures and prolonged incubation are required before this activity can be demonstrated[74]. From bovine lens the neutral proteinase has been partly purified, and it is

suggested that this enzyme might account for the entire *endo*peptidase activity of the lens[75]. The rates of hydrolysis by the bovine lens neutral proteinase have been measured with bovine crystallin fractions as substrates. The β_L-crystallin fraction was hydrolyzed at the highest rate, followed in decreasing order by α- and β_H-crystallin. γ-Crystallin was not hydrolyzed at all[76]. A similar neutral proteinase has been found in the human lens. In the human lens the activity of the neutral protease was only 25% of that determined in bovine lens[77].

Recently the hypothesis was put forward, that a variety of proteinases exist in lens tissue, but that their activities are normally controlled by specific inhibitors[78,79,80]. The group of Ortwerth was able to determine trypsin inhibitory activity in the bovine and rabbit lens. This trypsin-inhibitor activity was found almost exclusively in the water-soluble extracts of the cortical region of both bovine and rabbit lens, with little or no activity in extracts of nucleus or capsule. In the cortex extracts of the bovine lens these trypsin inhibitor activities were found in protein fractions with molecular weights of about 300,000 and 20,000[78]. A comparison between the trypsin inhibitor activities of the water-soluble and water-insoluble extracts of several zones of the bovine lens revealed that in the periphery of the lens all the inhibitor activity was in the water-soluble fraction, whereas towards the center of the lens (going to older fibers) the activity began shifting to the water-insoluble fraction. Comparable results were obtained from a comparison between prenatal, young and adult bovine lenses. Whether the insoluble fraction is active *in vivo* is not clear[81].

From the M_r 300,000 fraction an inhibitor was purified to homogeneity, which was effective in reducing the activity of trypsin, but complete inhibition was not seen even at high levels of inhibitor. However, a rapid and complete inhibition was observed with two *endo*genous trypsin-like proteinases isolated from the α-crystallin region fraction of bovine lens[79]. The trypsin inhibitor activity of bovine lens extracts could be completely inactivated *in vitro* by irradiation with long wavelength ultraviolet light of the extracts. This loss of trypsin inhibitor activity was accompanied by a breakdown of lens proteins[80].

In normal bovine and human water-soluble lens extracts the absence of these trypsin inhibitors in the lens nucleus can be correlated with the presence of trypsin-like proteolytic activity. In the cortical water-soluble extracts of bovine lenses trypsin-like activity could, however, be detected after a pre-

incubated period of 10-25 h at 34 °C in the presence of NaCl and $MgCl_2$[82]. Determination of the trypsin-like activity after activation in several zones of the bovine lens revealed that the total amount of enzyme activity gradually decreased from the lens outer cortex towards the lens nucleus. The capsule-epithelium and outer cortex had the highest specific activity. Separation of the trypsin-like activity on basis of size followed by activation revealed three molecular weight classes: 2.5×10^4, $6\text{-}7 \times 10^4$ and $7\text{-}9 \times 10^5$. From the class of M_r $7\text{-}9 \times 10^5$, which comigrated with the α-crystallin fraction, a low molecular weight proteinase was released after the activation period and cleavage of α-crystallin polypeptide chains was observed. The results suggest the release of proteinase activity from a complex between a trypsin-like enzyme and the *endo*genous lens inhibitor[83].

Bovine lens does not contain carboxypeptidases, but possesses a high activity of aminopeptidases[77,84]. The major portion of this activity in human and bovine lens is probably attributable to leucine aminopeptidase (EC 3.4.11.1), but also other aminopeptidases are present. Obvious candidates include alanine aminopeptidase (EC 3.4.11.14) and aminopeptidase B (EC 3.4.11.6)[72,85]. Determination of the aminopeptidase activity in several zones of the bovine lens revealed that these enzymes are found in all layers of the cortex and the nucleus, but not in the epithelium. From the outer cortex towards the nucleus a gradual decrease in enzyme activity is seen[86]. This activity has been ascribed to leucine aminopeptidase, but in view of the fact that also other aminopeptidases are present[72,85], it seems better to speak about the total aminopeptidase activity.

On basis of activity measurements it was concluded that very little aminopeptidase activity (including leucine aminopeptidase activity) was present in the human lens, when compared to calf lenses[72,73]. A recent study, which used micro-complement fixation tests, indicated that the concentration of LAP in aged human lenses is similar to that found in calf lenses. It is possible that the relatively low level of aminopeptidase activity observed in human lens homogenates is due to an age-related inactivation or degradation of LAP in human lenses, because only lenses from adult or elderly people were used[87].

There is no evidence for a direct *exo*peptidase activity of leucine aminopeptidase on lens crystallin fractions *in vitro*, even in the case of the γ-crystallins, which are non-acetylated[88]. Lens proteins constitute a very stable protein population. Ample evidence is available which supports a very slow protein turnover in lens as compared to other tissues[89,90]. Shortened

α-crystallin polypeptide chains, however, have been described. The proportion of the shortened chains increases from cortex to nucleus, suggesting an age-dependent process since no protein synthesis takes place any more in the inner cortex and the nucleus[91]. It has been proposed that the observed chains are the result of non-enzymatic breakage of chemically labile bonds[91,92]. It has been postulated from studies on the quaternary structure of bovine α-crystalline, by limited proteolysis, that the susceptible bonds are surface-exposed or become exposed as a result of age-related changes in secondary or tertiary structure[93]. The proteinases, activated by the elimination of inhibitors as already described above[78,79,80], might be able to nick these susceptible bonds. Anyhow, the peptide products of such degradations may well be broken down by the action of aminopeptidases (a.o. LAP), since these oligopeptides could not be detected[92]. Moreover, the C-terminal peptide of the αA-chain, cleaved by trypsin *in vitro*, remains non-covalently bound to the α-crystallin aggregate, but is nevertheless partly hydrolyzed by leucine aminopeptidase[93].

Although the reasons for the abundance of leucine aminopeptidase in bovine lens as well as its role in the intracellular protein degradation are still largely unclear, some relevant remarks can be made:

1. the high activities of aminopeptidases, of which the major portion is leucine aminopeptidase, and of activated trypsin-like proteases in the outer cortex, in comparison with other zones, point to a role in the intracellular breakdown of the protein-synthesizing apparatus[83,86]
2. because native crystallin fractions are poor substrates *in vitro*[88], it is likely that the normal substrates *in vivo* are shorter peptides, resulting from *endo*genous *endo*peptidase digestion[75]. This can be compared with the role of aminopeptidases in intracellular proteolysis as described for other mammalian cells[44,54,55,62,63].

AIM OF THE PRESENT INVESTIGATION

While the structure and reaction mechanism of many proteolytic enzymes are now known in detail, the aminopeptidases represent a class of enzymes still poorly understood. No x-ray structural analysis and no complete primary structure determination had hitherto been reported for any of the aminopeptidases. We have, therefore, undertaken an investigation of the primary struc-

ture of leucine aminopeptidase from bovine lens. The results of this investigation were obtained in close cooperation with Dr L.A.H. van Loon-Klaassen and have partly been described in her thesis, previously submitted to the University of Nijmegen[24].

In Chapter II the complete amino acid sequence of the polypeptide chain of leucine aminopeptidase is presented.

Chapter III describes the determination of the reactivity toward iodo-[2-^{3}H]acetate of the individual sulfhydryl groups in native leucine aminopeptidase and in the enzyme activated by metal ions. The enzymatic activities were determined of the products of Zn^{2+} ion readdition in the apoenzyme and the carboxymethylated apoenzyme. The chemical properties are discussed with regard to their possible functions in metal ion binding.

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Chapter II

THE PRIMARY STRUCTURE OF LEUCINE AMINOPEPTIDASE FROM BOVINE EYE LENS

SUMMARY

The amino acid sequence of bovine eye lens leucine aminopeptidase has been determined. Cyanogen bromide fragments, the COOH-terminal hydroxylamine fragment, and a large fragment obtained by digestion with *Staphylococcus aureus* protease were isolated from reduced and *S*-alkylated leucine aminopeptidase. The amino acid sequences of these fragments were determined by automated sequence analysis, by manual direct Edman degradation, and by the dansyl-Edman technique. Overlapping peptides were obtained by tryptic digestion of the *S*-alkylated protein or the citraconylated *S*-alkylated protein. The polypeptide chain of leucine aminopeptidase comprises 478 residues, corresponding to a molecular weight of 51,691. No significant sequence homology with any other published protein primary structure could be detected. This is the first report of a complete amino acid sequence of an enzyme belonging to the class of two-metal peptidases.

INTRODUCTION

Leucine aminopeptidase[1] is an *exo*peptidase catalyzing the hydrolysis of amino acids from the NH_2 terminus of polypeptide chains, and belongs to the class of aminopeptidases which are widely distributed in nature. Aminopeptidases with similar or identical properties have been found in many tissues, including lens, kidney, pancreas, muscle, and liver[1]. Among these, leucine aminopeptidase from bovine lens has been studied most extensively (see review by Hanson and Frohne[2]). Leucine aminopeptidase from bovine lens has a molecular weight of 326,000[3,4] and an isoelectric point of 4.9 ± 0.2[3]. It consists of six identical subunits of $M_r = 54{,}000$[4,5]. The crystalline enzyme contains two zinc

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[1] The enzymes used are: leucine aminopeptidase (EC 3.4.11.1), aminopeptidase M (EC 3.4.11.2), carboxypeptidase A (EC 3.4.17.1), carboxypeptidase B (EC 3.4.17.2), carboxypeptidase C (EC 3.4.17.-), carboxypeptidase Y (EC 3.4.17.-), chymotrypsin (EC 3.4.21.1), staphylococcal protease (EC 3.4.-.-), thermolysin (EC 3.4.24.4), trypsin (EC 3.4.21.4).

ions per subunit, one of which can easily be replaced by a manganese or magnesium ion, resulting in an increase in peptidase activity[6,7].

Electron microscopic investigations of leucine aminopeptidase in solution showed that the subunits are arranged at the vertices of a distorted triangular prism[8], and electron microscopic investigations of crystals of leucine aminopeptidase showed six asymmetric bilobal subunits arranged in such a way that the principal lobes are eclipsed and the minor lobes are staggered[9]. X-ray studies revealed that leucine aminopeptidase crystallizes in the hexagonal space group $P6_322$ with unit cell dimensions $a = b = 132$ Å and $c = 122$ Å[10]. Limited tryptic digestion of leucine aminopeptidase resulted in specific splitting of a single bond, while the leucine aminopeptidase aggregate remained intact[11,12].

Whereas the knowledge of the so-called one-metal peptidases (for instance, carboxypeptidases and thermolysin) has increased rapidly, the structure and reaction mechanism of the two-metal peptidases, to which class leucine aminopeptidase belongs, are still poorly understood[13]. No x-ray structural analysis of any of the two-metal peptidases is available as yet, and no primary structure of these enzymes has been published.

In the present paper, the complete amino acid sequence of the polypeptide chain of leucine aminopeptidase is presented. Part of the sequence, namely residues 1-171 comprising the NH_2-terminal cyanogen bromide fragment, has been reported in a preliminary communication[12]. The accompanying paper[14] describes the determination of the total number of sulfhydryl groups and their reactivity in the zinc metalloenzyme, in the enzyme activated by Mg^{2+}, Mn^{2+}, and Co^{2+}, and the metal-free enzyme.

EXPERIMENTAL PROCEDURES[2],[3]

Results

Most of the experimental evidence for the purification of fragments and for the determination of amino acid sequences is presented as a supplement immediately following the Discussion.[2]

Essentially pure leucine aminopeptidase was isolated from calf lenses by the method described. About 150 mg of enzyme was purified routinely from 800 lenses. Some preparations showed a minor band of $M_r = 20{,}000$ when analyzed by polyacrylamide gel electrophoresis in the presence of SDS. This protein, believed to be a γ-crystallin fraction on the basis of amino acid analysis, two-dimensional gel electrophoresis, and determination of the NH_2-terminal sequence (results not shown), could be removed only with extreme difficulty once the enzyme solution had been frozen and lyophilized. Purification could, however, very simply be achieved by chromatography of the native leucine aminopeptidase solution on Ultrogel AcA 34 in 0.1 M Tris-HCl buffer, pH 8.0. Purified enzyme revealed a single homogeneous peak in the ultracentrifuge (Fig. 1) and gave a single band both on SDS-containing polyacrylamide gels and on acidic urea gels (Fig. 2). The molecular weight of the subunit was

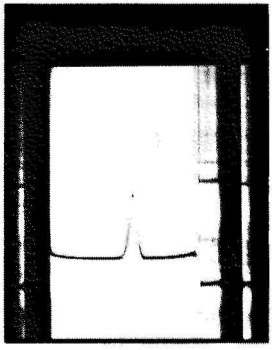

Fig. 1 Sedimentation velocity analysis of leucine aminopeptidase. Sedimentation studies were performed at 20 °C with native enzyme in 0.1 M Tris-HCl buffer, pH 8.0, at 64,000 rpm.

estimated to be 54,000. After activation by manganese ions, the hydrolytic activity of the leucine aminopeptidase solution was determined. Optimum manganese concentration for activation was 1 mM. Kinetic parameters were de-

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[2] The 'Experimental Procedures' and part of the 'Results' including Figs 5 to 35 and Tables II to XLII (see page 69 to 84) are presented as supplement at the end of this chapter.

[3] The abbreviations used are: CNBr, cyanogen bromide; HA, hydroxylamine; SDS, sodium dodecyl sulfate.

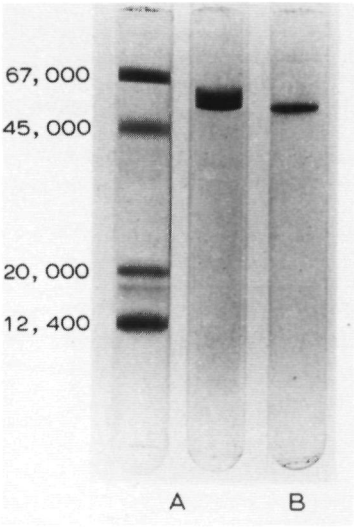


Fig. 2 Electrophoretic analysis of leucine aminopeptidase. A, SDS-polyacrylamide gel electrophoresis of purified leucine aminopeptidase from bovine lens. Left: molecular weight marker proteins. Right: purified leucine aminopeptidase. Gels were stained with Coomassie brilliant blue. B: acidic urea gel electrophoresis of purified leucine aminopeptidase. Gel was stained with amido black.

termined using a series of six substrate concentrations ranging from 20 to 100 mM. The Michaelis constant for the Mn^{2+}-activated leucine aminopeptidase was determined to be 29.4 mM and V_{max} was 1.35 mmol/min/mg of enzyme.

The amino acid composition of leucine aminopeptidase is presented in Table I. For comparison, the values reported by Carpenter and Vahl[6] and by Kettmann *et al*[15] are included. The enzyme contains a high percentage of hydrophobic amino acids. Values found for tryptophan vary considerably. We used three different methods for the tryptophan determination, but were not able to obtain reproducible results. However, none of the values determined exceeded 4.0. The number of tryptophan residues deduced from the sequence results is 6. The NH_2-terminal residue of native and of *S*-carboxymethylated leucine aminopeptidase was determined to be threonine. By automated Edman degradation, the NH_2-terminal amino acid sequence of leucine aminopeptidase was determined up to Thr-21 (Table II).

The general strategy for the sequence determination of leucine aminopeptidase is outlined in Fig. 3. Cleavage by CNBr at the 12 methionine residues of reduced and *S*-alkylated enzyme yielded 13 fragments, of which 11 fragments could be purified partially or completely. We were not able to purify fragments CB 7 and CB 13. It is conceivable that these fragments precipitate during gel filtration or ion exchange chromatography. Fragments CB 8 and CB 11 were isolated as a mixture, but fragment CB 11 could be obtained in pure form after CNBr cleavage of hydroxylamine fragment HA 2. A large part of the sequence of fragment CB 8 was deduced from fragment HA 2-CB 8b purified from a

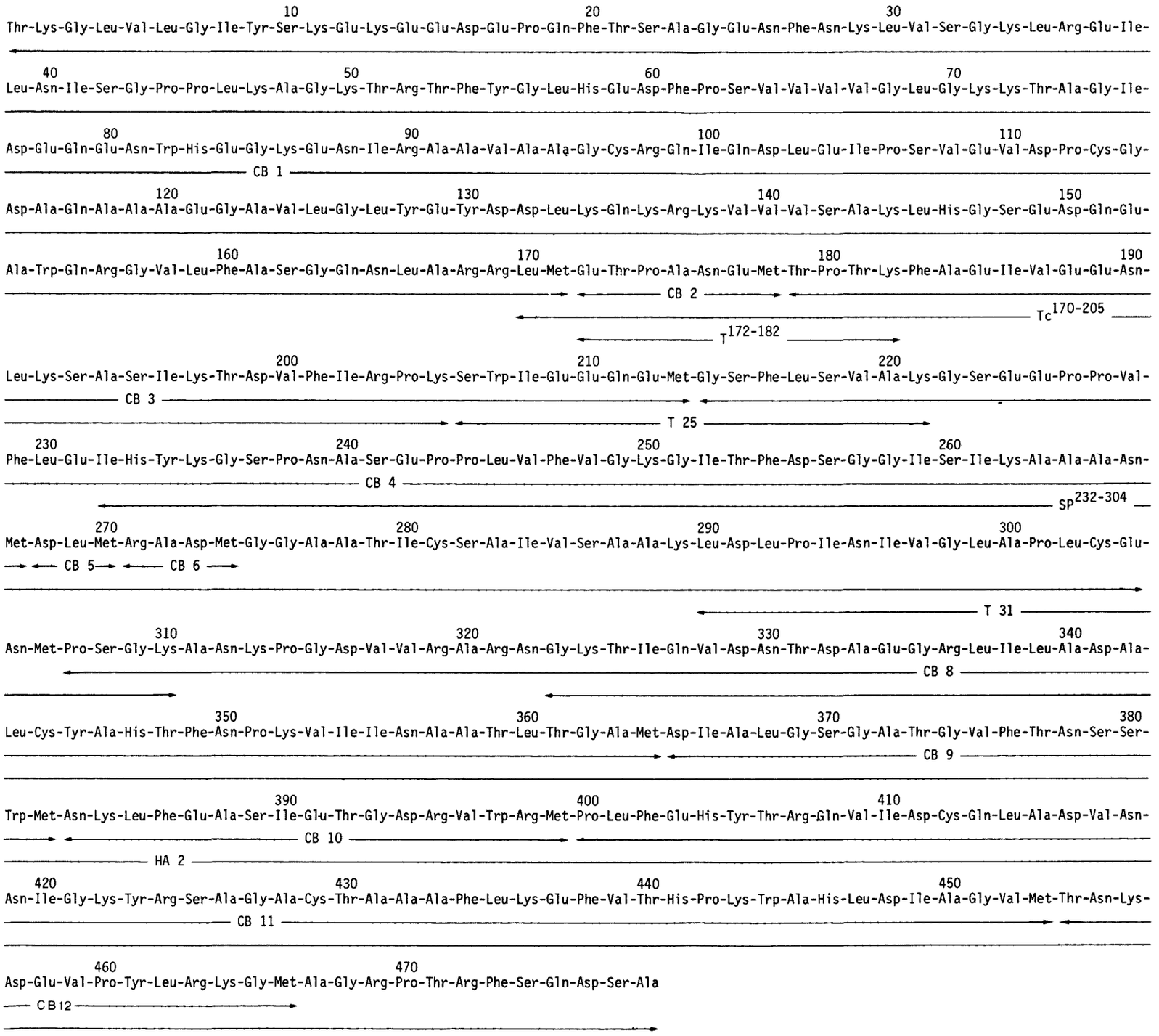
Table I *The amino acid composition of bovine lens leucine aminopeptidase.* Values for leucine aminopeptidase are expressed as residues per subunit. Results are the average of triplicate analyses. Values for valine and isoleucine were taken from the 72 h hydrolysate, and values for threonine and serine were extrapolated to zero time hydrolysis. (A) reduced and *S*-carboxymethylated leucine aminopeptidase, (B) sequence results, (C) values taken from Carpenter and Vahl[6], (D) values taken from Kettmann *et al*[15]

Amino acid	(A)	(B)	(C)	(D)	
Aspartic acid	46.5	47[b]	47	42.8	
Threonine	24.1	24	25	22.3	
Serine	29.2	29	28	26.2	
Glutamic acid	52.3	50[c]	52	48.2	
Proline	23.8	23	26	25.0	
Glycine	42.3	42	42	38.3	
Alanine	52.5	53	53	48.0	
Cysteine	6.8[a]	7	8	11.8	(8)[d]
Valine	33.3	33	33	29.5	
Methionine	11.2	12	11	9.4	(10)[d]
Isoleucine	27.4	28	29	24.6	
Leucine	38.3	36	39	35.0	
Tyrosine	8.9	9	10	8.2	
Phenylalanine	19.2	18	20	17.7	
Histidine	8.9	8	8	7.3	(8)[d]
Lysine	36.1	33	34	31.1	
Arginine	22.4	20	21	19.2	
Tryptophan	3.8	6	10	6.0	(7)[d]
Total	487.0	478	496	450.6	
M_r	52,799.7	51,691	54,060	48,878	

[a]Determined as *S*-carboxymethylcysteine
[b]25 aspartic acid and 22 asparagine
[c]36 glutamic acid and 14 glutamine
[d]values between brackets are improved values[2]

See opposite site:

Fig. 4 *Proposed amino acid sequence of bovine lens leucine aminopeptidase.* Determination of the sequence of the complete polypeptide chain was performed as outlined in the schematic representation (Fig. 3).


10 20 30
Thr-Lys-Gly-Leu-Val-Leu-Gly-Ile-Tyr-Ser-Lys-Glu-Lys-Glu-Glu-Asp-Glu-Pro-Gln-Phe-Thr-Ser-Ala-Gly-Glu-Asn-Phe-Asn-Lys-Leu-Val-Ser-Gly-Lys-Leu-Arg-Glu-Ile-
40 50 60 70
Leu-Asn-Ile-Ser-Gly-Pro-Pro-Leu-Lys-Ala-Gly-Lys-Thr-Arg-Thr-Phe-Tyr-Gly-Leu-His-Glu-Asp-Phe-Pro-Ser-Val-Val-Val-Val-Gly-Leu-Gly-Lys-Lys-Thr-Ala-Gly-Ile-
80 90 100 110
Asp-Glu-Gln-Glu-Asn-Trp-His-Glu-Gly-Lys-Glu-Asn-Ile-Arg-Ala-Ala-Val-Ala-Ala-Gly-Cys-Arg-Gln-Ile-Gln-Asp-Leu-Glu-Ile-Pro-Ser-Val-Glu-Val-Asp-Pro-Cys-Gly-
CB 1
120 130 140 150
Asp-Ala-Gln-Ala-Ala-Ala-Glu-Gly-Ala-Val-Leu-Gly-Leu-Tyr-Glu-Tyr-Asp-Asp-Leu-Lys-Gln-Lys-Arg-Lys-Val-Val-Val-Ser-Ala-Lys-Leu-His-Gly-Ser-Glu-Asp-Gln-Glu-
160 170 180 190
Ala-Trp-Gln-Arg-Gly-Val-Leu-Phe-Ala-Ser-Gly-Gln-Asn-Leu-Ala-Arg-Arg-Leu-Met-Glu-Thr-Pro-Ala-Asn-Glu-Met-Thr-Pro-Thr-Lys-Phe-Ala-Glu-Ile-Val-Glu-Glu-Asn-
CB 2
Tc170-205
T172-182
200 210 220
Leu-Lys-Ser-Ala-Ser-Ile-Lys-Thr-Asp-Val-Phe-Ile-Arg-Pro-Lys-Ser-Trp-Ile-Glu-Glu-Gln-Glu-Met-Gly-Ser-Phe-Leu-Ser-Val-Ala-Lys-Gly-Ser-Glu-Glu-Pro-Pro-Val-
CB 3
T 25
230 240 250 260
Phe-Leu-Glu-Ile-His-Tyr-Lys-Gly-Ser-Pro-Asn-Ala-Ser-Glu-Pro-Pro-Leu-Val-Phe-Val-Gly-Lys-Gly-Ile-Thr-Phe-Asp-Ser-Gly-Gly-Ile-Ser-Ile-Lys-Ala-Ala-Ala-Asn-
CB 4
SP232-304
270 280 290 300
Met-Asp-Leu-Met-Arg-Ala-Asp-Met-Gly-Gly-Ala-Ala-Thr-Ile-Cys-Ser-Ala-Ile-Val-Ser-Ala-Ala-Lys-Leu-Asp-Leu-Pro-Ile-Asn-Ile-Val-Gly-Leu-Ala-Pro-Leu-Cys-Glu-
CB 5
CB 6
T 31
310 320 330 340
Asn-Met-Pro-Ser-Gly-Lys-Ala-Asn-Lys-Pro-Gly-Asp-Val-Val-Arg-Ala-Arg-Asn-Gly-Lys-Thr-Ile-Gln-Val-Asp-Asn-Thr-Asp-Ala-Glu-Gly-Arg-Leu-Ile-Leu-Ala-Asp-Ala-
CB 8
350 360 370 380
Leu-Cys-Tyr-Ala-His-Thr-Phe-Asn-Pro-Lys-Val-Ile-Ile-Asn-Ala-Ala-Thr-Leu-Thr-Gly-Ala-Met-Asp-Ile-Ala-Leu-Gly-Ser-Gly-Ala-Thr-Gly-Val-Phe-Thr-Asn-Ser-Ser-
CB 9
390 400 410
Trp-Met-Asn-Lys-Leu-Phe-Glu-Ala-Ser-Ile-Glu-Thr-Gly-Asp-Arg-Val-Trp-Arg-Met-Pro-Leu-Phe-Glu-His-Tyr-Thr-Arg-Gln-Val-Ile-Asp-Cys-Gln-Leu-Ala-Asp-Val-Asn-
CB 10
HA 2
420 430 440 450
Asn-Ile-Gly-Lys-Tyr-Arg-Ser-Ala-Gly-Ala-Cys-Thr-Ala-Ala-Ala-Phe-Leu-Lys-Glu-Phe-Val-Thr-His-Pro-Lys-Trp-Ala-His-Leu-Asp-Ile-Ala-Gly-Val-Met-Thr-Asn-Lys-
CB 11
460 470
Asp-Glu-Val-Pro-Tyr-Leu-Arg-Lys-Gly-Met-Ala-Gly-Arg-Pro-Thr-Arg-Phe-Ser-Gln-Asp-Ser-Ala
CB12

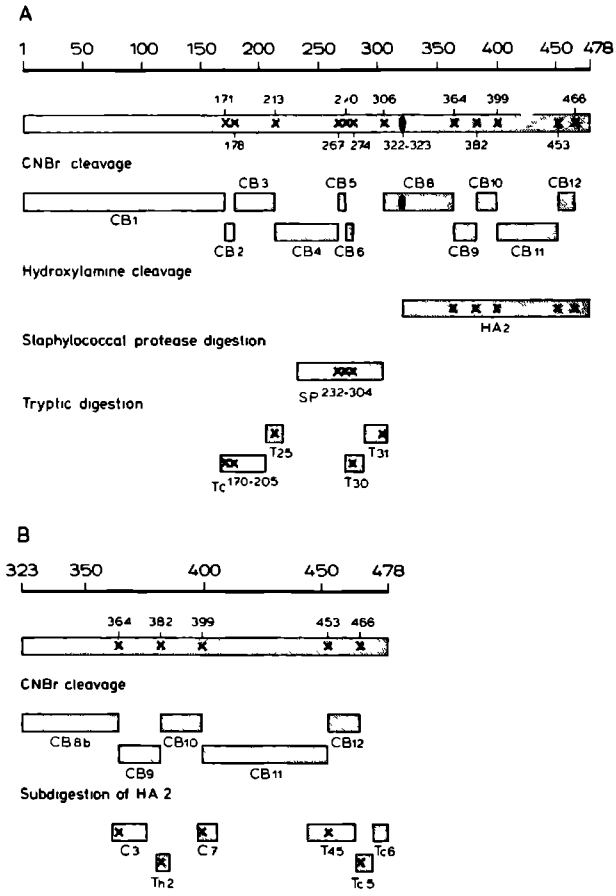


Fig. 3 Schematic representation of the strategy for sequence analysis of bovine lens leucine aminopeptidase. Sites of cleavage are indicated by the following symbols: X, methionine and ●, Asn-Gly. A: determination of the sequence of the complete polypeptide chain was performed by purification and sequence analysis of cyanogen bromide fragments, of the COOH-terminal hydroxylamine fragment, and of the large staphylococcal protease peptide $SP^{232-304}$. Overlaps between these fragments were obtained from tryptic peptides and from peptide $Tc^{170-205}$, which resulted from a tryptic digest of citraconylated leucine aminopeptidase. B: determination of the sequence of the COOH-terminal hydroxylamine fragment (HA 2). Cyanogen bromide fragments of HA 2 were purified and sequenced. The COOH-terminal sequence (residues 466-478) was deduced from tryptic peptides Tc 5 and Tc 6, obtained from citraconylated HA 2. Overlapping peptides were isolated by digestion of HA 2 with trypsin, chymotrypsin, and thermolysin.

CNBr cleavage mixture of fragment HA 2. Treatment of leucine aminopeptidase with hydroxylamine resulted in cleavage of the only Asn-Gly bond in the chain

(Fig 3B). CNBr cleavage of the COOH-terminal fragment HA 2 yielded five fragments in pure form (CB 8b, CB 9, CB 10, CB 11, and CB 12). Overlaps between these fragments were obtained from tryptic, chymotryptic, thermolytic, and staphylococcal protease peptides of HA 2. Tryptic digestion of citraconylated HA 2 yielded two peptides, Tc 5 and Tc 6. They originated from the COOH-terminal part of HA 2 and contain sequences which were never found in a CNBr fragment. Peptide HA 2-Tc 6 was identified as the COOH-terminal peptide of HA 2 and hence of the complete leucine aminopeptidase chain. The COOH-terminal alanine residue could not be identified by digestion with carboxypeptidases A, B,C, or Y, even when the digestions were performed under denaturing conditions, such as in the presence of SDS or urea, and by heat denaturation. Probably the COOH-terminal residues of leucine aminopeptidase are shielded in such a way that they are not accessible to the carboxypeptidases. All overlaps between the CNBr-fragments have been rigidly established. Tryptic digestion of *S*-carboxymethylated leucine aminopeptidase and purification of methionine-containing peptides T 25, T 30, and T 31 together with the purification of the tryptic peptide $Tc^{170-205}$, obtained from a digest of citraconylated *S*-carboxymethylated leucine aminopeptidase, established the remaining overlaps. The sequence of residues 275 to 304 was derived from analysis of peptide $SP^{232-304}$, obtained from a staphylococcal protease digestion of *S*-aminoethylated leucine aminopeptidase (Fig. 3A). The sequence of $SP^{232-304}$ also confirmed the order of fragments CB 4, CB 5, and CB 6.

The proposed amino acid sequence of leucine aminopeptidase is presented in Fig. 4 (page 32-33).

In contrast to earlier reports[16] proposing that bovine lens leucine aminopeptidase is made up of 10 subunits with molecular weights of 32,000, it is now firmly established that the enzyme consists of six identical subunits of M_r = 54,000[4,5]. We have never found any indication of a component of leucine aminopeptidase smaller than the M_r = 54,000 subunit; not even on prolonged incubation of the carboxymethylated subunit at 37 °C or in SDS and β-mercaptoethanol. This contradicts the claims that prolonged incubation of the subunits in 1% SDS and 1% β-mercaptoethanol results in the appearance of smaller components, or even that polypeptide chains of M_r = 10,000 are present in the aggregate[17]. The results of cross-linking studies by Carpenter and Harrington[5], which indicated that leucine aminopeptidase is composed of identical subunits, are confirmed by our findings that the subunits are identical both in molecular weight and in charge, and by the fact that we never found any evidence for heterogeneities in the primary structure during our sequence studies.

Comparison of the total amino acid composition, derived from the sequence results, with the amino acid analysis of leucine aminopeptidase reveals very good agreement. The results of our amino acid analysis correspond reasonably well with the values reported by Kettmann *et al*[15] and Carpenter and Vahl[6] (Table I). Only for tryptophan is a significant discrepancy observed. Although we determined the tryptophan content of leucine aminopeptidase by three different methods, we were not able to obtain reproducible results. A total of 6 tryptophan residues was deduced from the sequence results.

Initial attempts to isolate CNBr fragments were hampered by incomplete cleavage, insoluble core material, and considerable aggregation between fragments. These problems were solved by extensive reduction and alkylation of leucine aminopeptidase prior to cleavage by CNBr. Purification of CNBr fragments could be achieved only by ensuring complete solubilization of these polypeptides during chromatographic manipulations. All separations were therefore carried out in solvents containing 6 M urea. Despite these precautions, we were still unable to purify CNBr fragments CB 7 and CB 13. All amino acid residues in the sequence of leucine aminopeptidase were identified more than once in peptides obtained by different methods. All overlaps between larger fragments could be established conclusively, apart from the overlap between residues 472 and 473, which was confirmed by an indirect method, based on the

normal specificity of trypsin and the lack of lysine or arginine in Tc 6.

The polypeptide chain of leucine aminopeptidase contains a total of 478 amino acid residues. The calculated molecular weight is 51,691. However, polyacrylamide gel electrophoresis in the presence of SDS resulted in an estimated molecular weight of 54,000. The discrepancy of 2300 between the two values is of the same magnitude as that found for CNBr fragment CB 1: a value of 18,837 was calculated from the sequence results, while a value of 21,000 was estimated from polyacrylamide gel electrophoresis in the presence of SDS[12]. It may well be that the NH_2-terminal part of leucine aminopeptidase binds relatively less SDS than the remaining part of the polypeptide chain[18].

The amino acid sequence of bovine lens leucine aminopeptidase proposed here represents the first primary structure determination of a member of the class of two-metal peptidases. To detect sequence relationships between leucine aminopeptidase and other proteins, six pieces of 25 residues from the total sequence of 478 residues were compared with all 25-residue segments of each sequence in the data base of the Atlas of Protein Sequence and Structure, using the computer program SEARCH[19]. No statistically significant relationship was found with any sequence in the data base, indicating that leucine aminopeptidase represents a separate protein superfamily.

The sequence of leucine aminopeptidase can provide a basis for the interpretation of x-ray crystallographic studies, of which a preliminary account has been presented[10]. It has also been useful in interpreting experiments involving the affinity labeling of cysteine residues in metallo-activated leucine aminopeptidase, as presented in the following paper[14]. These results may provide a better understanding of the mechanism of action on a molecular basis of this class of enzyme.

EXPERIMENTAL PROCEDURES

Preparation of leucine aminopeptidase from bovine lens. Leucine aminopeptidase (LAP)[1] was isolated from batches of eight hundred calf lenses according to the method of Hanson and Frohne[2]. Occasionally further purification was necessary. In that case the enzyme solution, in 0.1 M Tris-HCl buffer, pH 8.0, was centrifuged at 55,000 rpm in a Spinco centrifuge (Ti 60 rotor). The supernatant fraction was concentrated in a Diaflo Ultrafilter (Amicon XM50) to approximately 5 ml, and chromatographed on a column (120 x 3.0 cm) of Ultrogel AcA 34 (LKB), eluted with 0.1 M Tris-HCl buffer, pH 8.0, at a flow rate of 18 ml/h. Purity was checked routinely by polyacrylamide gel electrophoresis in the presence of SDS[18]. Protein concentration was determined by the method of Lowry[20]. Acidic urea gel electrophoresis was carried out as described earlier[11,12]. Sedimentation studies were performed at 20 °C in a Beckman Spinco model E analytical ultracentrifuge at 64,000 rpm, using Schlieren optics. Activity measurement and determination of the kinetic parameters were carried out as described previously[11]. For sequence studies the enzyme solution was dialyzed against water and lyophilized.

Reduction and S-alkylation. After reduction, the cysteine residues of LAP were modified by *S*-carboxymethylation or *S*-aminoethylation prior to further cleavage. Because of resistance of LAP against *S*-alkylation the special procedure of Melbye and Carpenter[4] with longer reduction time and larger excess of alkylating reagent was followed. Lyophilized LAP (40 mg) was reduced for 4 h by β-mercaptoethanol in 8 M urea. The pH was brought to 8.5 with 4 M NaOH, and 280 mg of iodoacetic acid (Sigma), neutralized with 4 M NaOH, was added. The reaction was allowed to proceed for 10 min under nitrogen, while the pH was kept constant with 1 M NaOH. The solution was dialyzed against 0.01 M

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[1] The abbreviations used are: Ae-LAP, reduced and *S*-aminoethylated leucine aminopeptidase; Cm-LAP, reduced and *S*-carboxymethylated leucine aminopeptidase; dansyl-, 5-dimethylaminonaphthalene-1-sulfonyl-; EGTA; Ethyleneglycol-bis-(β-aminoethylether)N,N,N', N'-tetraacetic acid; LAP, leucine aminopeptidase; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Quadrol, N,N,N', N'-tetrakis(2-hydroxypropyl)ethylene diamine.

Tris-HCl, pH 8.5, and water, and then lyophilized.

Labeling with [2-^{3}H]-iodoacetic acid (Radiochemical Centre, Amersham) was carried out on 40 mg of LAP by reduction and alkylation with 37 µmol of neutralized [2-^{3}H]-iodoacetic acid (sp. act. 10.8 µCi/µmol), which corresponds to a 6-fold molar excess over cysteine residues. After extensive dialysis and lyophilization, the [^{3}H]-labeled protein was once more reduced and alkylated with 280 mg of iodoacetic acid as described above to assure complete alkylation of cysteine residues.

Reduction and *S*-aminoethylation was performed according to Raftery and Cole[21] in 1.0 M Tris-HCl buffer, pH 8.6, using a reduction time of 4 h.

Citraconylation of ε-amino groups. Citraconylation was performed according to Atassi and Habeeb[22] with slight modifications. 40 mg of protein was dissolved in 4 ml of 6 M guanidine-HCl containing 0.5 mM EGTA. The pH was brought to 8.2 with 4 M NaOH and 230 µl of citraconic acid anhydride (Pierce) (150-fold molar excess based on the number of lysine residues) was added in portions over a period of 30 min, while the pH was maintained at 8.2 with 1 M NaOH. After 90 min the solution was extensively dialyzed against 0.1 M NH_4HCO_3, pH 8.9. Subsequently the citraconylated protein was digested with trypsin (Worthington TRTPCK) at 37 °C for 2 h, using 1% (w/w) of trypsin initially and an additional 1% after 1 h. Trypsin was inactivated by heating the incubation mixture for 4 min at 90 °C. The lyophilized peptide mixture was subjected to gel filtration in 0.1 M ammonia or 0.05 M NH_4HCO_3. Decitraconylation was obtained by incubation in 10% (v/v) acetic acid for 2 h at 37 °C.

CNBr cleavage. CNBr cleavage[23] was performed in 70% (v/v) trifluoro-acetic acid at a protein concentration of 50 mg/ml and a CNBr concentration of 200 mg/ml for 18 h at room temperature in the dark. The solution was then diluted with 10 volumes of 50% (v/v) acetic acid and concentrated by rotary evaporation. The residue was diluted with water and lyophilized. Purification of the fragments was obtained by ion-exchange chromatography on Cm-cellulose (Whatman, CM-52) in the presence of 7 M urea[24] or by gel filtration on Sephadex G-100 fine (200 x 3.5 cm) eluted at a flow rate of 10 ml/h with 10% (v/v) acetic acid, containing 6 M urea. The urea was removed by gel filtration on Sephadex G-10 in 20% (v/v) acetic acid.

Hydroxylamine cleavage. Hydroxylamine cleavage of *S*-alkylated LAP was carried

out according to Bornstein and Balian[25] with some modifications. Hydroxylamine (Fluka; 13.9 g) was dissolved in 8 ml of water. Under vigorous stirring 8 ml of 12.5 M NaOH was added in an icebath, followed by 20 ml of 1 M K_2CO_3. The pH was adjusted to 9.5 with 4 M NaOH. Guanidine-HCl (57.3 g) was added, and the volume brought to 120 ml by the addition of water. After readjustment of the pH to 9.5, *S*-alkylated LAP was added to a final concentration of 4 mg/ml. After a reaction time of 4 h at 25 °C the mixture was desalted by extensive dialysis against water, and lyophilized. The cleavage mixture was purified and fractionated by gel filtration on Sephadex G-100 fine (200 x 3.5 cm), eluted with 10% (v/v) acetic acid, containing 6 M urea, at a flow rate of 10 ml/h. Pooled fractions were dialyzed against water and lyophilized.

Enzymic digestions. Digestion of the *S*-alkylated protein or purified fragments with trypsin (Worthington TRTPCK), chymotrypsin (Worthington CBI) or thermolysin (Calbiochem A grade) was carried out in 0.1 M NH_4HCO_3, pH 8.9. Tryptic- and chymotryptic digestion was performed for 2 h at 37 °C at a protein concentration of 10 mg/ml using 1% (w/w) of enzyme initially and an additional 1% after 1 h. Thermolytic digestion was carried out for 1 h at 50 °C at a protein concentration of 10 mg/ml using 1% (w/w) of enzyme. Occasionally fragments were first oxidized with performic acid[26] before thermolytic digestion in order to enhance the yield of the peptides. Chymotryptic or thermolytic digestion of tryptic peptides was carried out for 1 h at 37 °C at a peptide concentration of 500 nmol/ml, using 5 µl of enzyme solution (10 mg/ml) per ml of incubation mixture. Digestion of CNBr and hydroxylamine fragments by staphylococcal protease (Miles, *S. aureus* V8) was carried out for 16 h at 37 °C in 0.1 M NH_4HCO_3, pH 8.0, at a protein concentration of 20 mg/ml using 5% (w/w) of enzyme. During the digestion of Ae-LAP and *S*-aminoethylated HA-2 fragment 2 M urea was included in the digestion buffer. Digestion of peptides with staphylococcal protease was performed for 6 h at 40 °C in 0.1 M NH_4HCO_3, pH 8.0, at a peptide concentration of 0.5 mg/ml using 5% (w/w) enzyme[27]. Digestion with aminopeptidase M (Boehringer) was carried out at 37 °C for 25 h in 0.1 M NH_4HCO_3, pH 8.0, at a peptide concentration of 50 nmol/100 µl, using 10 µl of enzyme solution in a 100 µl incubation volume. The lyophilized digestion mixture was applied directly to the amino acid analyzer. Digestion with carboxypeptidase A and B (Boehringer) was performed at 37 °C in 0.1 M N-ethylmorpholine acetate buffer, pH 8.5, according to Am-

bler[28] and was also tested in the presence of 0.1% (w/v) or 1% (w/v) SDS. After a digestion time of 4 h the pH was lowered to 5.3 with acetic acid and digestion was continued with carboxypeptidase C as described by Tschesche[29]. Digestion with carboxypeptidase Y from baker yeast (Pierce) was performed as described by Hayashi and was also tried out on enzyme denatured during 10 min at 100 °C and in the presence of 6 M urea[30]. All digestions were stopped by freezing and lyophilization, unless stated otherwise.

Purification of peptides. Peptides resulting from enzymic digestion of small fragments were purified by high-voltage electrophoresis and descending chromatography as described earlier[31]. Aspartic acid was used as a reference for the determination of the mobility of the peptides, and their charge was calculated according to Offord[32]. When separation of neutral peptides was unsatisfactory, re-electrophoresis was performed at pH 3.5 in pyridine/glacial acetic acid/water (1:10:89, v/v). Peptides were eluted from paper with 10% (v/v) acetic acid, or 0.1 M ammonia in the case of peptides containing an *N*-terminal glutamine residue that can undergo cyclization. Occasionally maps were stained with Ehrlich's reagent for the detection of peptides containing tryptophan[33]. Peptides resulting from enzymic digestion of large fragments, like CB 1 and HA 2, were first separated on a column (120 x 1.5 cm) of Sephadex G-50 superfine in 0.05 M NH_4HCO_3 or 0.1 M ammonia. Maximum sample loading was 40 mg of digestion mixture. Peptides were eluted at a flow rat of 6 ml/h and detected by their absorbance at 280 and 206 nm (Uvicord III, LKB). Large peptides were further purified by ion-exchange chromatography on a column (10.0 x 1.0 cm) of DEAE-cellulose (Whatman DE-52)[34]. Peptides were eluted with a gradient (2 x 200 ml) from 0.05 M to 0.3 M NH_4HCO_3, pH 7.8, at a flow rate of 12 ml/h. Smaller peptides were further purified by peptide mapping as described above. Occasionally peptides containing methionine were isolated by the diagonal electrophoretic method of Tang and Hartley[35].

Amino acid analysis and sequence determination. Protein samples were hydrolyzed for 24, 48 and 72 h in 0.5 ml of 6 M HCl containing 0.025% (w/v) phenol in sealed, evacuated tubes at 110 °C. Peptide samples were hydrolyzed for 22 h at 110 °C. Amino acid analysis was performed with a Rank Hilger Chromaspek analyzer. Tryptophan was determined after hydrolysis with 3 M *p*-toluenesulfonic acid[36], by the colorimetric method of Basha and Roberts[37], or as described by Messineo and Musarra[38].

The NH_2-terminal amino acid residue of larger fragments was determined by dansylation according to Gros and Labouesse[39], and in the case of peptides by the method described by Gray[40]. The dansyl-Edman technique was performed according to Gray and Smith[41] using sequencing reagent from Pierce (Sequenal grade). Dansylated amino acids were identified by thin-layer chromatography on polyamide sheets (Schleicher and Schüll, F1700, 5 x 5 cm). The solvent systems of Woods and Wang[42] were used, modified by replacing benzene by toluene in solvent II. For identification of dansyl-histidine the system of Jörnvall[43] was used.

For direct manual Edman degradation the accelerated version of Niall and Potts[44] was followed. For some of the lysine peptides the Braunitzer reagent 4-sulphophenylisothiocyanate (Pierce) was used in the first step[45]. The thiazolinone derivatives were converted with 1 M HCl for 10 min at 80 °C, and the phenylthiohydantoins were extracted three times with ethylacetate. The phenylthiohydantoins in the organic phase were identified by thin-layer chromatography on silicagel HPTLC plates (Merck, 10 x 10 cm, cat. nr. 5628) containing a fluorescence indicator. The plates were developed with chloroform/ethanol (98:2, v/v) followed by chloroform/methanol (90/10, v/v) in the same direction. After visualization of the spots at 254 nm[46], the plates were stained with ninhydrin-collidine reagent[47]. The phenylthiohydantoins were quantified by high performance liquid chromatography on a column (250 x 4.6 nm) of Nucleosil 50-5 (Schleicher and Schüll) as described by Frank and Strubert[48] For the elution of the phenylthiohydantoins of the hydrophobic amino acids a solvent consisting of methylene chloride/methanol (99.75:0.25, v/v) was used, and for the elution of the phenylthiohydantoins of the hydrophylic amino acids methylene chloride/methanol/dimethyl sulfoxide (95:5:3, v/v). The phenylthiohydantoins of aspartic acid and glutamic acid were only identified by thin layer chromatography. For the identification of the phenylthiohydantoins of histidine and arginine the aqueous phase was subjected to chromatography on the above mentioned silicagel plates, developed with xylene/95% ethanol/acetic acid (50:50:0.5, v/v)[49]. After irradiation at 254 nm the plates were stained with Pauli's reagent[33].

Automated Edman degradation was performed in a Beckman spinning cup sequenator, model 890C, using the quadrol double cleavage program. The phenylthiohydantoins were quantified by gas liquid chromatography as described[50].

Amide groups were assigned on the basis of electrophoretic mobilities of small peptides at pH 6.5 or by direct identification as phenylthiohydantoins

in the Edman degradation. Occasionally amide groups were identified by amino acid analysis following digestion with aminopeptidase M.

Peptide nomenclature. Fragments resulting from chemical cleavage are indicated by prefices corresponding to the type of cleavage: CB = cyanogen bromide, HA = hydroxylamine, and are numbered according to their position in the complete chain. Tryptic peptides are indicated by T and numbered starting from the NH_2-terminus of the complete polypeptide chain.

Designations for the other peptides are: Tc, tryptic digestion after citraconylation; C, chymotryptic digestion; Th, thermolytic digestion and SP, staphylococcal protease digestion. These peptides are numbered starting from the NH_2-terminus of the fragment from which they were isolated. Sometimes a peptide is encoded with a superscript indicating the NH_2-terminal and the COOH-terminal residue number in the over-all sequence of LAP.

Computer analyses. Computer analyses were performed by Dr W.C. Baker, Georgetown University Medical Center, Washington D.C. with the computer program SEARCH[19], which compares a segment of 25 residues to all possible segments of the same length in sequences accumulated in 'Atlas of Protein Sequence and Structure'; Volume 5. Six segments of 25 residues of LAP were investigated.

RESULTS[2] [3]

Purification of the CNBr fragments of reduced and S-alkylated leucine aminopeptidase. The CNBr cleavage mixture of Cm-LAP was chromatographed on Sephadex G-100 in 10% (v/v) acetic acid containing 6 M urea (Fig. 5A). The SDS-polyacrylamide gel electrophoretic analysis of the different fractions is shown in Fig. 5B. The first peak (pool II) yielded a fragment which was puri-

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[2] General legends to the Figures:
Identifications of residues were performed by the following methods:
notation (⟶⟶), automatic Edman degradation
notation (⟶), manual Edman degradation
notation (⟶), dansyl Edman degradation
notation (⟶), manual- and dansyl Edman degradation.

Elution pattern; absorbance was measured at two wavelengths, at 206 nm (——) and at 280 nm (---).

[3] Tables II to XLII are presented in miniprint at page 69 to 84 .

fied completely by re-chromatography on the same column. Its molecular weight was estimated to be 21,000. Pool III and pool IV contained mixtures of fragments with molecular weights of approximately 10,000 and 6,000, respectively. Fractions eluting later from the column contained the smaller fragments.

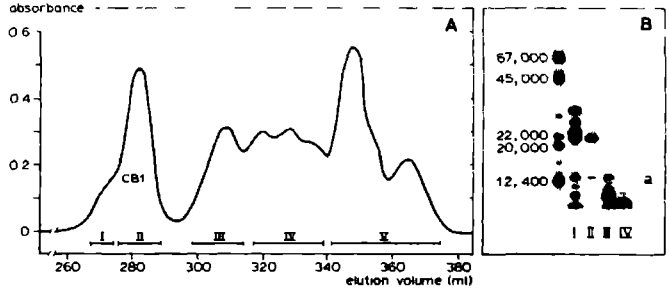

Fig. 5A Chromatography of the CNBr fragments of S-carboxymethylated LAP. Approximately 300 mg of lyophilized fragments was applied to a column (200 x 3.5 cm) of Sephadex G-100 fine, and eluted with 10% (v/v) acetic acid containing 6 M urea at a flow rate of 10 ml/h. Fractions were pooled as indicated. Absorbance was measured at 280 nm.

Fig. 5B SDS-polyacrylamide gel electrophoresis of the pooled fractions of the Sephadex-column.
Pool I-IV of the Sephadex G-100 column of the separation of the CNBr fragments were analyzed by SDS-polyacrylamide gel electrophoresis.

A sequenator run of the 21,000 molecular weight fragment gave essentially the same results as for native LAP (Table II), indicating that this fragment is the NH_2-terminal fragment of the enzyme (CB 1).

Some CNBr fragments were purified from Ae-LAP by chromatography on Cm-cellulose in the presence of 7 M urea (Fig. 6) followed by gel filtration on Sephadex G-50 superfine.

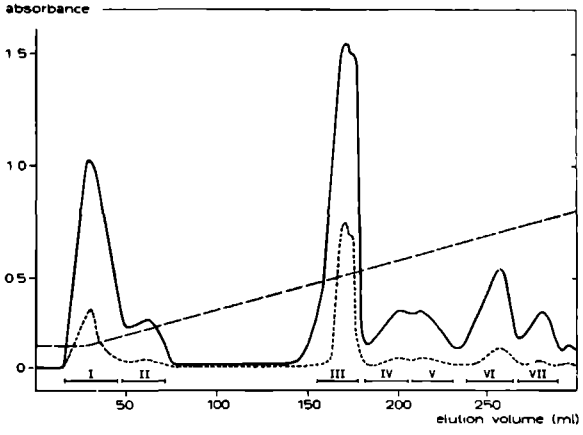

Fig. 6 Ion-exchange chromatography of the CNBr fragments of Ae-LAP. Approximately 100 mg of lyophilized CNBr fragments was applied to a column (10.0 x 2.0 cm) of Cm-cellulose in 0.01 M Na_2HPO_4, pH 5.8, containing 7 M urea, and eluted by means of a linear gradient made of 200 ml starting buffer and 200 ml of limit buffer (0.04 M Na_2HPO_4, pH 8.0). Fractions were pooled as indicated. Pools I and II were purified by gel filtration (Fig. 7).

Chromatography of pool I and II of the Cm-cellulose column (Fig. 6) gave identical results (Fig. 7). Pool I of the Sephadex column (Fig. 7) contained

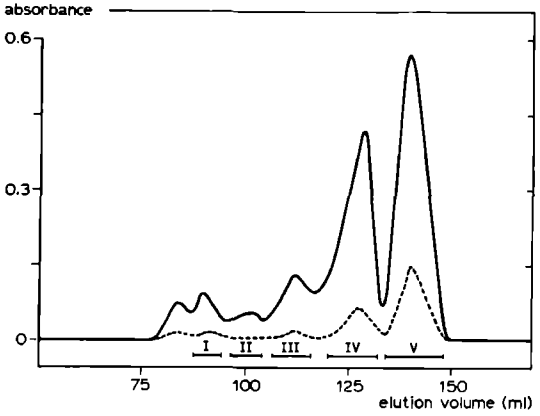

Fig. 7 Purification of CNBr fragments of Ae-LAP. Pool I of the Cm-cellulose column (Fig. 6) was applied to a column (120 x 1.6 cm) of Sephadex G-50 superfine in 20% (v/v) acetic acid and eluted with the same solvent at a flow rate of 8 ml/h. Fractions were pooled as indicated. Pool III was further purified by gel filtration (Fig. 8) and pool V was purified by peptide mapping (Fig. 9A).

an almost pure fragment CB 3-4 resulting from incomplete cleavage at methionine residue 213. Pool II of the Sephadex column (Fig. 7) contained a mixture of fragments resulting from incomplete cleavage at methionine residues 267 and 270. These fragments were not purified. Re-chromatography of pool III of the Sephadex column (Fig. 7) on the same column, but eluted with 5% instead of 20% (v/v) acetic acid yielded two peaks (Fig. 8). The first peak

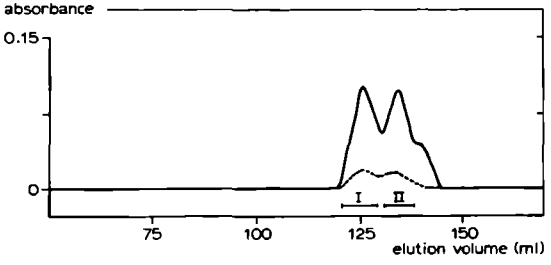

Fig. 8 Purification of CNBr fragments of Ae-LAP. Pool III of the Sephadex G-50 column (Fig. 7) was applied to a column (115 x 1.6 cm) of Sephadex G-50 superfine in 5% (v/v) acetic acid and eluted with the same solvent at a flow rate of 6 ml/h. Fractions were pooled as indicated.

(pool I) yielded fragment CB 4 in pure form. Pool II contained a mixture of two fragments, CB 4 and CB 2-3, resulting from incomplete cleavage at methionine residue 178. Pool IV of the Sephadex column (Fig. 7) contained CB 3 in pure form. Peptide mapping of pool V of the Sephadex column (Fig. 7) yielded two fragments: CB 9 and CB 10 (Fig. 9A). Pool III of the Cm-cellulose column (Fig. 6) contained a mixture of fragment CB 1 and larger fragments, which resulted from incomplete cleavage at methionine bonds. This fraction was not further purified. Analysis of pool IV and V of the Cm-cellulose column (Fig. 6) showed the presence of a reasonably pure fragment CB 8. Although this frag-

ment could not be purified completely, we were able to determine its amino acid sequence. Pool VI of the Cm-cellulose column (Fig. 6) contained a mixture of fragments CB 8 and CB 11, and pool VII contained almost pure fragment CB 11. We did not try to purify these pool fractions, because fragment CB 11 could be isolated from a CNBr cleavage mixture of fragment HA 2. Be-

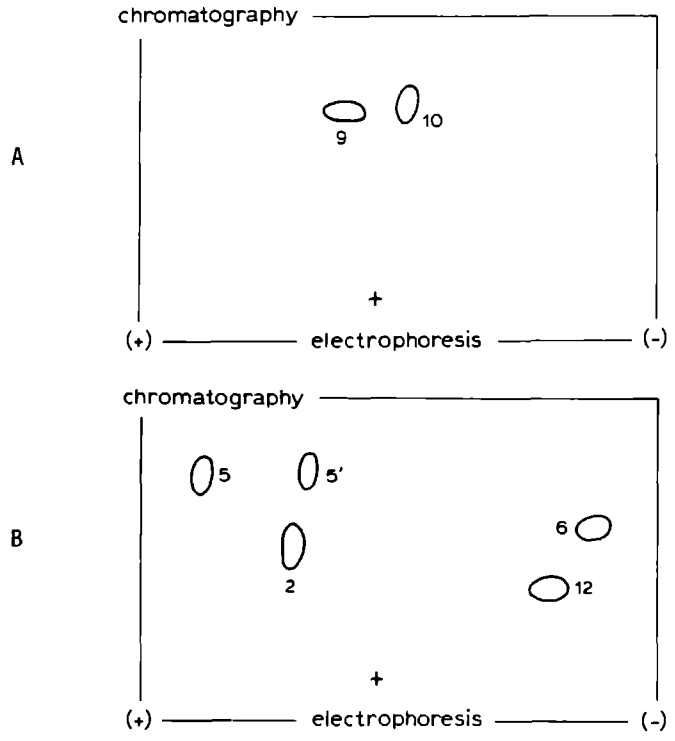

Fig. 9 Purification of CNBr fragments of reduced and S-alkylated LAP by peptide mapping.

A. Pool V of the Sephadex column (Fig. 7) was applied at the origin (+) and subjected to high-voltage electrophoresis at pH 6.5 followed by descending chromatography in the second dimension.

B. Approximately 12 mg of lyophilized CNBr fragments of Cm-LAP was applied at the origin (+) and subjected to high-voltage electrophoresis at pH 6.5 followed by descending chromatography in the second dimension.

cause the small CNBr fragments were lost during removal of urea by desalting on Sephadex G-10, these fragments were purified by direct peptide mapping of a CNBr cleavage mixture of Cm-LAP (Fig. 9B). This yielded four pure fragments: CB 2, CB 5, CB 6 and CB 12.

The amino acid compositions of the purified CNBr fragments are listed in Table III.

Sequence analysis of fragment CB 1. Fragment CB 1 of Cm-LAP was digested with trypsin and the resulting peptides were fractionated directly on Sephadex G-50 superfine (Fig. 10). Pool I yielded the large peptide T 14 in pure form. Pool II was purified by ion-exchange chromatography on DEAE-cellulose (Fig. 11). From this column T 9 was obtained in pure form. The smaller peptides, present in pool III (Fig. 10), were purified by peptide mapping. Peptide T 2 could not be recovered, probably because of its hydrophobic nature, but its sequence could be deduced from the sequenator run on total CB 1 (Table II). Two lysine bonds and one arginine bond were partially split by trypsin, resulting in peptides T 10-11, T 16-17 and T 20-21. The amino acid compositions of the purified tryptic peptides of CB 1 are listed in Table IV. Peptides T 14a and T 14b were purified from a tryptic digest of CB 1 isolated from Ae-LAP. The sequence determination of the tryptic peptides is shown in Fig. 12*. Some tryptic peptides were subdigested with thermolysin or chymotrypsin in order to confirm or complete the sequence. The amino acid compositions of

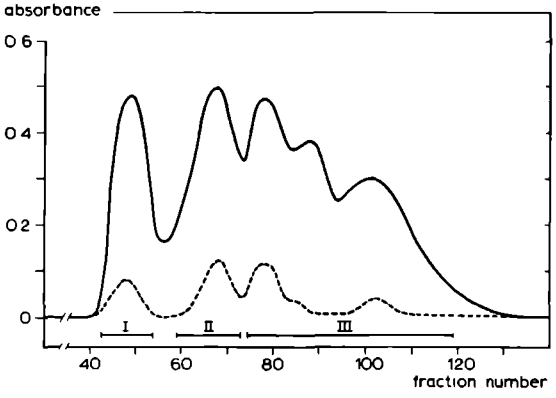

Fig. 10 Fractionation of the tryptic peptides of CB 1 on Sephadex G-50.
A 40 mg sample of the tryptic digestion mixture of CB 1 was applied to a column (120 x 1.5 cm) of Sephadex G-50 superfine and eluted with 0.1 M ammonia at a flow rate of 5 ml/h. Fractions of 2.0 ml were collected and pooled as indicated. Pool I yielded peptide T 14 in pure form. Pool II was further purified by ion-exchange chromatography (Fig. 11), and pool III was purified by peptide mapping.

these secondary peptides are listed in Table V. The lysine bond (res. 13-14) was not cleaved by trypsin, probably because of several adjacent acidic residues. The sequence determination of the large peptide T 14 will be described in the section 'Purification and sequence determination of the cysteine-containing tryptic peptides of LAP'. Peptide T 15 comprised a Lys-Arg bond which was not cleaved by trypsin. Two peptides containing a homoserine residue were found as a result of a partial splitting of an arginine bond (res. 169-170) by trypsin.

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*See page 48-49.

The order of tryptic peptides in CB 1 was determined by means of peptides obtained by chymotryptic and thermolytic digestion of CB 1 and by tryptic digestion of citraconylated CB 1. Chymotryptic peptides of CB 1 were purified by gel filtration on Sephadex G-50 superfine followed by peptide mapping as described before[12]. The amino acid compositions of the purified chymotryptic peptides are listed in Table VI. The sequence determination of the chymo-

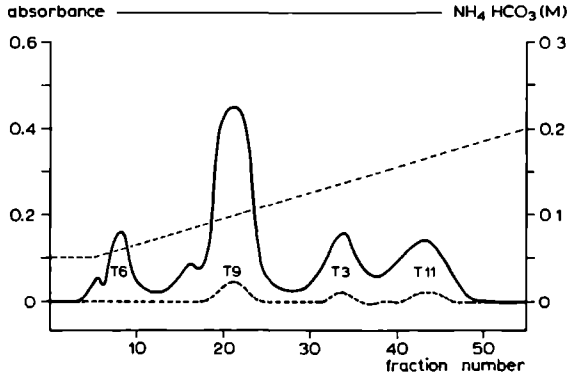
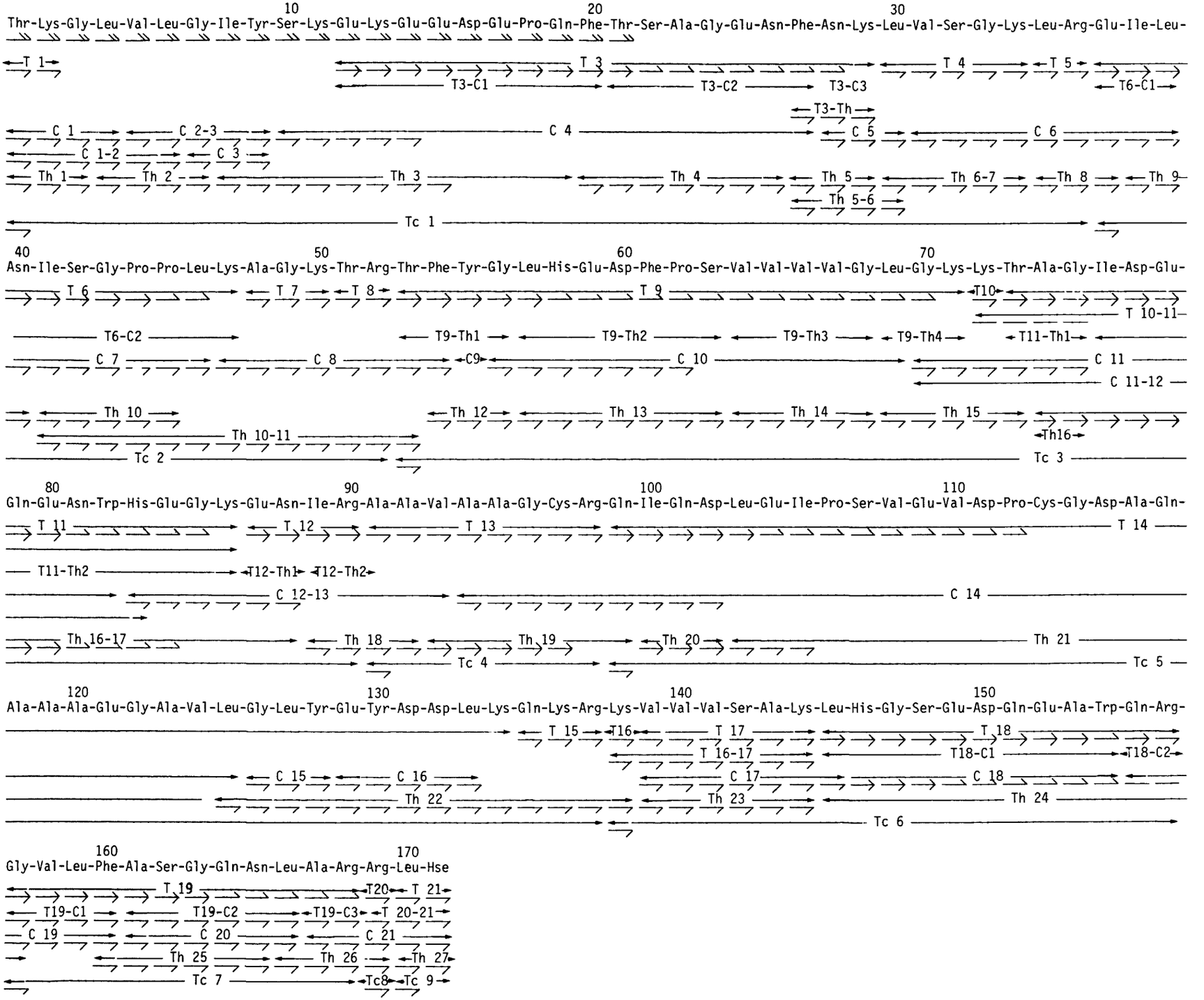


Fig. 11 Purification of tryptic peptide T 9 of CB 1 by ion-exchange chromatography on DEAE-cellulose.
Pool II of the Sephadex G-50 column (Fig. 10) was dissolved in 0.05 M NH_4HCO_3, pH 7.8, and brought onto a column (11.0 x 1.0 cm) of DE-52 equilibrated with the same buffer. Peptides were eluted with a gradient from 0.05 to 0.3 M NH_4HCO_3, pH 7.8, at a flow rate of 12 ml/h. Fractions of 4.0 ml were collected.

See opposite site:

Fig. 12 Proposed amino acid sequence of fragment CB 1.
The amino acid sequence of peptide T 14 is presented in Fig. 18.


10
20
30
Thr-Lys-Gly-Leu-Val-Leu-Gly-Ile-Tyr-Ser-Lys-Glu-Lys-Glu-Glu-Asp-Glu-Pro-Gln-Phe-Thr-Ser-Ala-Gly-Glu-Asn-Phe-Asn-Lys-Leu-Val-Ser-Gly-Lys-Leu-Arg-Glu-Ile-Leu-
T 1
T 3
T 4
T 5
T3-C1
T3-C2
T3-C3
T6-C1
T3-Th
C 1
C 2-3
C 4
C 5
C 6
C 1-2
C 3
Th 1
Th 2
Th 3
Th 4
Th 5
Th 6-7
Th 8
Th 9
Th 5-6
Tc 1
40
50
60
70
Asn-Ile-Ser-Gly-Pro-Pro-Leu-Lys-Ala-Gly-Lys-Thr-Arg-Thr-Phe-Tyr-Gly-Leu-His-Glu-Asp-Phe-Pro-Ser-Val-Val-Val-Val-Gly-Leu-Gly-Lys-Lys-Thr-Ala-Gly-Ile-Asp-Glu-
T 6
T 7
T 8
T 9
T10
T 10-11
T6-C2
T9-Th1
T9-Th2
T9-Th3
T9-Th4
T11-Th1
C 7
C 8
C9
C 10
C 11
C 11-12
Th 10
Th 12
Th 13
Th 14
Th 15
Th16
Th 10-11
Tc 2
Tc 3
80
90
100
110
Gln-Glu-Asn-Trp-His-Glu-Gly-Lys-Glu-Asn-Ile-Arg-Ala-Ala-Val-Ala-Ala-Gly-Cys-Arg-Gln-Ile-Gln-Asp-Leu-Glu-Ile-Pro-Ser-Val-Glu-Val-Asp-Pro-Cys-Gly-Asp-Ala-Gln-
T 11
T 12
T 13
T 14
T11-Th2
T12-Th1
T12-Th2
C 12-13
C 14
Th 16-17
Th 18
Th 19
Th 20
Th 21
Tc 4
Tc 5
120
130
140
150
Ala-Ala-Ala-Glu-Gly-Ala-Val-Leu-Gly-Leu-Tyr-Glu-Tyr-Asp-Asp-Leu-Lys-Gln-Lys-Arg-Lys-Val-Val-Ser-Ala-Lys-Leu-His-Gly-Ser-Glu-Asp-Gln-Glu-Ala-Trp-Gln-Arg-
T 15
T16
T 17
T 18
T 16-17
T18-C1
T18-C2
C 15
C 16
C 17
C 18
Th 22
Th 23
Th 24
Tc 6
160
170
Gly-Val-Leu-Phe-Ala-Ser-Gly-Gln-Asn-Leu-Ala-Arg-Arg-Leu-Hse
T 19
T20
T 21
T19-C1
T19-C2
T19-C3
T 20-21
C 19
C 20
C 21
Th 25
Th 26
Th 27
Tc 7
Tc8
Tc 9

tryptic peptides is included in Fig. 12*. The amino acid compositions of thermolytic peptides of CB 1 purified by peptide mapping are given in Table VII, and their sequence determination is shown in Fig. 12. The peptide mixture obtained by tryptic digestion of citraconylated CB 1 was chromatographed on Sephadex G-50 superfine and after decitraconylation fractions were purified by ion-exchange chromatography or peptide mapping as described before[12]. The amino acid compositions of these peptides are given in Table VIII. Their NH_2-terminal residues were determined by dansylation. In some cases the peptides were again digested with trypsin after decitraconylation, purified and analyzed (see Fig. 12*).

The accumulated evidence for sequence determination of CB 1 is shown in Fig. 12*. Most overlaps between tryptic peptides involved two or more residues. The overlap of T 3 and T 4 involves only one residue, but the order of these peptides was confirmed by tryptic digestion of peptide Tc 1, which yielded T 1 to T 5, and by the amino acid composition of Tc 1. The order T 15-T 16-T 17 could not be proved by the chymotryptic or thermolytic peptides, because the Lys-Val bond (res. 138-139) was split by both enzymes. However, citraconyl peptide Tc 6, with NH_2-terminal lysine, clearly indicated the order of these peptides. Fragment CB 1 contains a total of 171 residues. The amino acid composition of this fragment (Table III) is in good agreement with the results obtained from the sequence determination.

Sequence analysis of fragment CB 3. All tryptic peptides of fragment CB 3 were purified by peptide mapping. The amino acid compositions of the tryptic peptides are listed in Table IX. Chymotryptic digestion of CB 3 followed by peptide mapping yielded only two peptides (Table IX). Thermolytic digestion of CB 3 yielded six peptides which were purified by peptide mapping. The amino

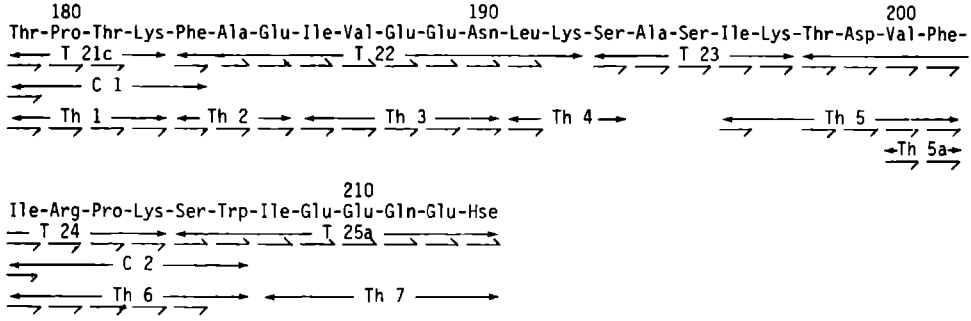


Fig. 13 Proposed amino acid sequence of fragment CB 3

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*Page 48-49.

acid compositions of these peptides are shown in Table X. The evidence for the sequence determination of fragment CB 3 is summarized in Fig. 13. The overlaps T 21c-T 22 and T 22-T 23 both involve only a single residue, but the order T 21c-T 22-T 23 is established conclusively by the comparison of the amino acid composition of CB 3 with the number of residues of the particular amino acids obtained from the sequence determination (Table III).

Sequence analysis of fragment CB 4. Peptide mapping of a tryptic digest of fragment CB 4 yielded all peptides in pure form. The amino acid analyses of the tryptic peptides are presented in Table XI. Peptide T 27 was partially modified by deamidation of residue 239 (about 30%) during purification. Chymotryptic and thermolytic peptides of CB 4 were purified by peptide mapping. The amino acid compositions are shown in Tables XII and XIII. The sequence determination of the purified peptides, together with the total sequence of fragment CB 4 is shown in Fig. 14. The overlap T 28-T 29a only involved one residue, but because peptide T 29a is the only tryptic peptide of CB 4 with NH_2-terminal alanine, T 29a must follow T 28.

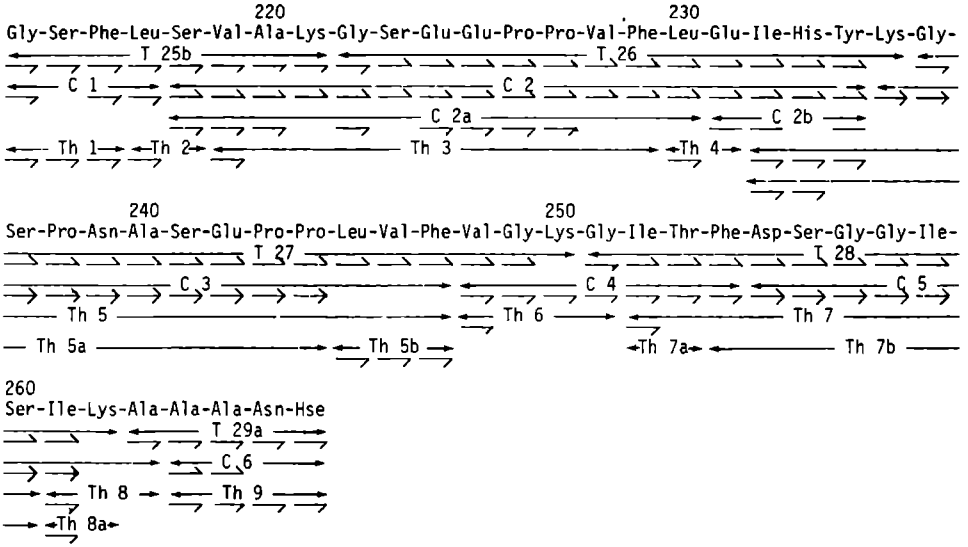

Fig. 14 Proposed amino acid sequence of fragment CB 4.

Sequence analysis of fragment CB 8. Fragment CB 8 of the Ae-LAP could not be obtained in completely pure form and a correct amino acid analysis of CB 8 can therefore not be presented. Tryptic digestion followed by peptide mapping

yielded the purified peptides T 31b through T 36b and some minor impurities. The compositions of these purified peptides are given in Table XIV. Peptide mapping of chymotryptic and thermolytic digests of this fragment yielded peptides of which the amino acid analyses are presented in Tables XV and XVI, respectively. Sequence determination of these peptides and evidence for the alignment are given in Fig. 15. These results confirm the sequence of resi-

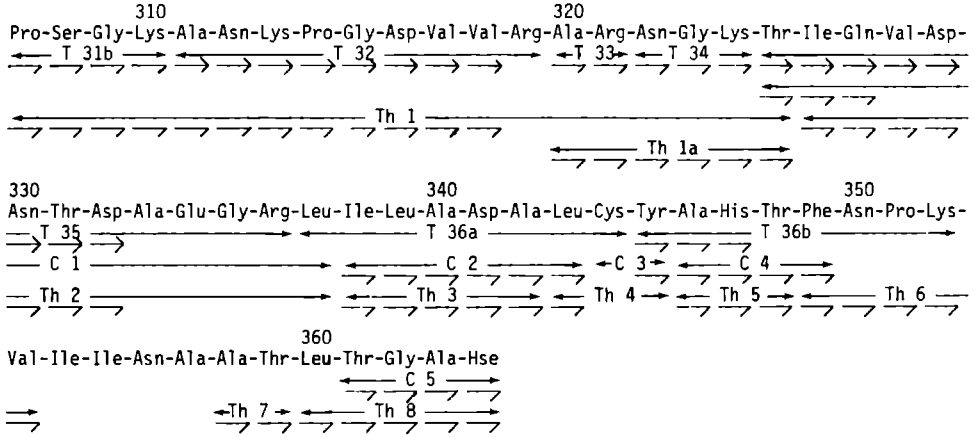

Fig. 15 Proposed amino acid sequence of fragment CB 8.
These results confirm the sequence of residues 307-324 and the alignment of T 31b to T 34. Additional results for the remaining part of the sequence are presented in Fig. 24.

dues 307-324 and the alignment of peptides T 31b to T 34. The remaining part of the sequence of this fragment was deduced from fragment HA 2-CB 8b (see section 'Sequence analysis of fragment HA 2-CB 8b').

Sequence analysis of the CNBr fragment CB 2, CB 5, CB 6, CB 9, CB 10 and CB 12. Direct manual Edman degradation of fragment CB 2 enabled to identify the first six residues (Fig. 16). The assignment of the COOH-terminal homoserine was based on the amino acid composition of the fragment (Table III). The amino acid sequence of fragments CB 5 and CB 6 was determined by dansyl-Edman degradation (Fig. 16). Eight residues of fragment CB 9 were successfully identified by direct manual Edman degradation. A chymotrypic digest of CB 9 yielded two peptides CB 9-C 1 and CB 9-C 2 (Table XVII). Because only six sequence steps could be performed on CB 9-C 1 it was subdigested with thermolysin (Table XVII). The sequence of the purified peptides, together with the sequence of CB 9-C 2 and the amino acid composition of the fragment complet-

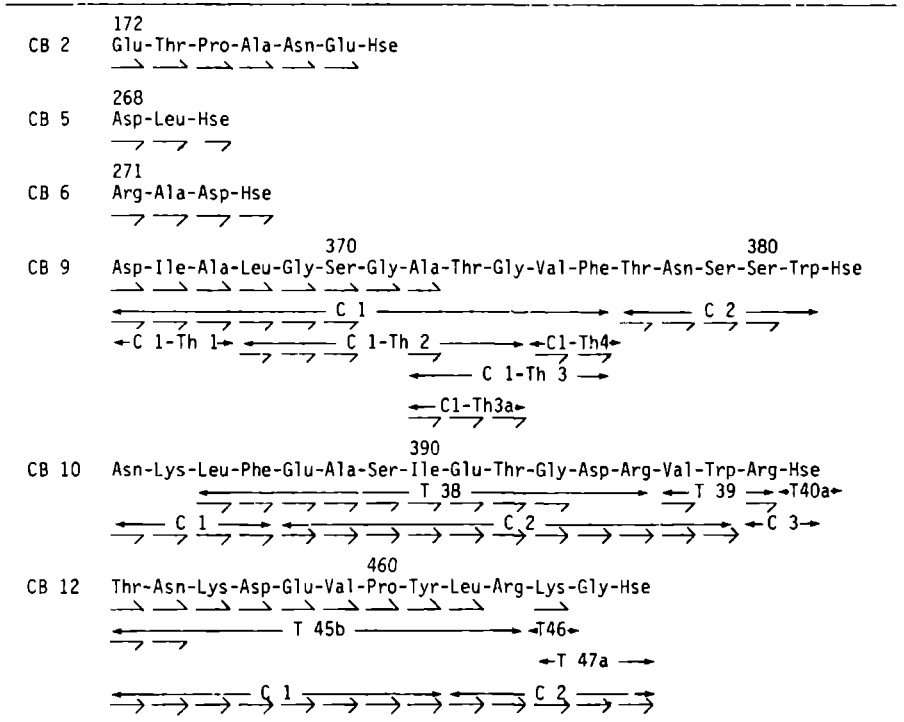

Fig. 16 Amino acid sequence determination of the CNBr fragments CB 2, CB 5, CB 6, CB 9, CB 10 and CB 12.

ed the amino acid sequence of CB 9 (Fig. 16). Purification of tryptic and chymotryptic peptides of CB 10 (Table XVIII), followed by sequence determination of these peptides yielded the sequence of fragment CB 10 (Fig. 16). The amino acid sequence of fragment CB 12 was determined up to the penultimate residue by direct manual Edman degradation (Fig. 16). The sequence was confirmed by the amino acid composition and partial sequence determination of tryptic and chymotryptic peptides (Table XIX; Fig. 16).

Purification and sequence determination of the cysteine-containing tryptic peptides of LAP. To determine the overlaps around cysteine residues and between CNBr fragments, we purified the cysteine-containing tryptic peptides from [2-^{3}H]-Cm-LAP. Most of the cysteine-containing peptides were purified from the *S*-aminoethylated fragments and this approach yielded additional cleaving sites for trypsin. A tryptic digest of [2-^{3}H]-Cm-protein was applied

to a Sephadex G-50 superfine column (Fig. 17). All the incorporated radioactivity was associated with *S*-carboxymethylcysteine residues. Pool I yielded peptide T 14 in pure form. The following fractions were analyzed by peptide

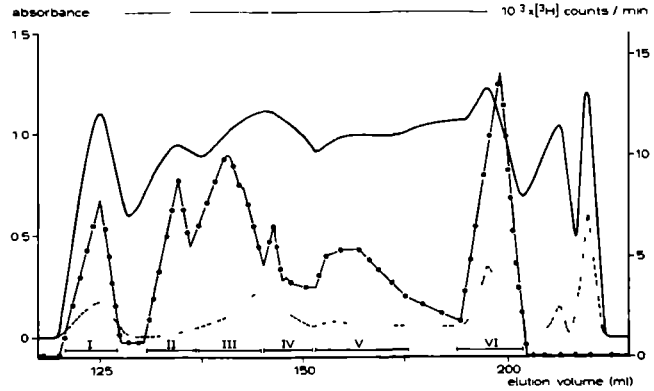

Fig. 17 Purification of the cysteine-containing tryptic peptides of LAP. A tryptic digestion mixture of [2-^{3}H]-Cm-protein was applied to a Sephadex G-50 superfine column (120 x 1.6 cm) in 0.1 M ammonia and eluted with the same solvent at a flow rate of 6 ml/h. Fractions of 2 ml were collected. From each fraction 50 µl was traced for radioactivity (—●—●—●—). Fractions were pooled as indicated. Pool II up to VI were further purified by peptide mapping.

mapping. Pool II contained peptide T 31, pool III T 30 and T 41, pool IV T 36, pool V T 43 and pool VI T 13. The amino acid compositions of the cysteine-containing tryptic peptides are listed in Table XX. For completion and confirmation of their sequences, peptide T 14, T 31 and T 41 were subdigested with either chymotrypsin or thermolysin and/or staphylococcal protease. The amino acid compositions of the peptides obtained from subdigestion are presented in Table XXI. Evidence for the sequence determination of these peptides is given in Fig. 18. In the case of peptide T 14 the sequence could be deduced from peptide T 14a and T 14b, which were purified from a tryptic digest of fragment CB 1 of Ae-LAP (Table XXII). The amino acid sequences of T 14, T 30, T 31 and T 41 complete the sequences of fragments CB 1, $SP^{232-304}$ and CB 11.

Purification of the COOH-terminal hydroxylamine fragment (HA 2) of reduced and S-alkylated LAP. Treatment of S-alkylated LAP with hydroxylamine generated two major cleavage products of apparent molecular weights of 39,000 and 15,000, respectively (Fig. 19). After a cleavage time of 4 h the cleavage percentage was about 50%. The mixture was chromatographed on a Sephadex G-100 column in 10% (v/v) acetic acid containing 6 M urea (Fig. 20) and the pool

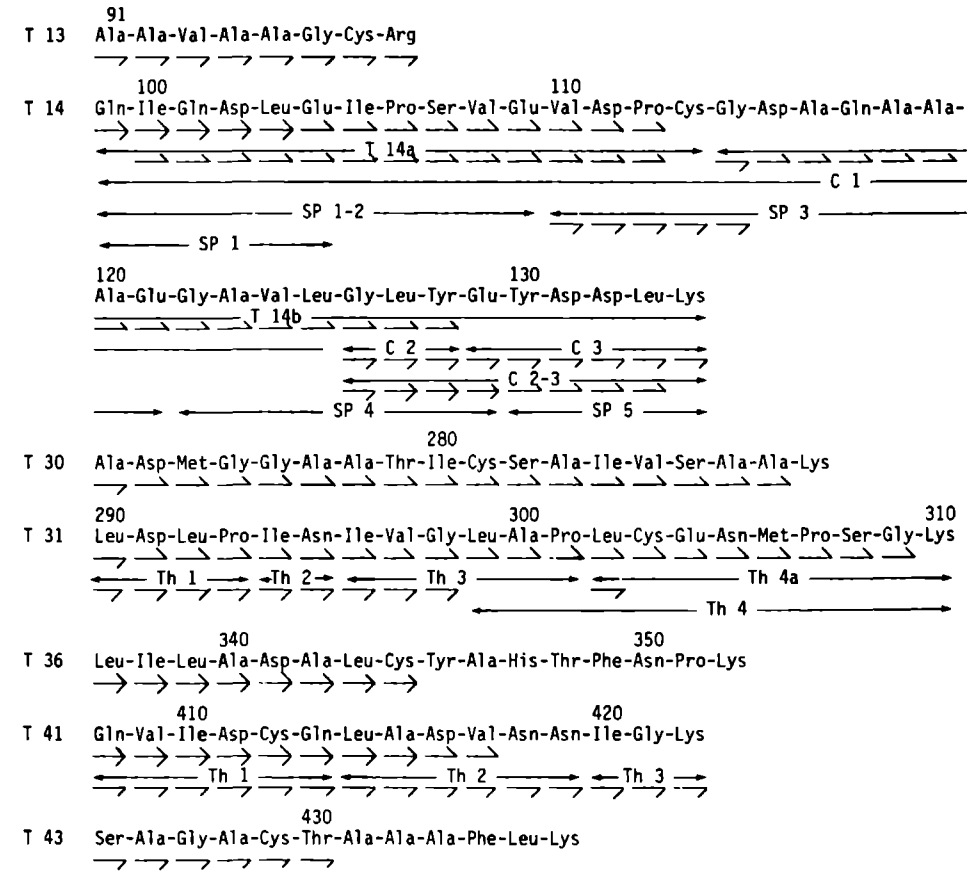

Fig. 18 Amino acid sequence determination of the cysteine-containing tryptic peptides of LAP.

fractions were analyzed by SDS gel electrophoresis (Fig. 19). Determination of the NH_2-terminal residue of pool I and II yielded threonine and of pool IV glycine, which proved the 39,000 dalton fragment to be the NH_2-terminal fragment (HA 1), and the 15,000 fragment the COOH-terminal part of the chain (HA 2). Pool IV (Fig. 20) obtained from Ae-LAP could be purified further by ion-exchange chromatography on Cm-cellulose (Fig. 21) in the presence of 7 M urea. Pool I (Fig. 21) contained unidentificable fragments. The NH_2-terminal residue found in pool II was threonine, and analysis of a tryptic digest showed only peptides which were also isolated from fragment CB 1, including peptide T 1. We concluded that pool II contained a product of non-specific

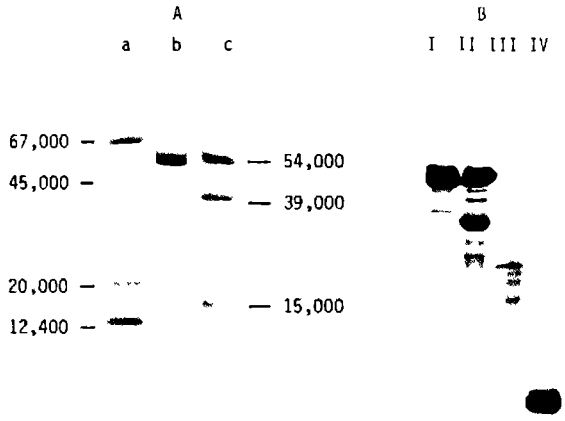

Fig. 19A SDS-polyacrylamide gel electrophoresis of S-alkylated LAP after cleavage by hydroxylamine.
a) Marker proteins; b) *S*-alkylated LAP; c) hydroxylamine treated *S*-alkylated LAP.
B SDS-polyacrylamide gel electrophoresis of the pooled fractions of the Sephadex G-100 column (Fig. 20).
Pool I-IV of the Sephadex G-100 column of the separation of the hydroxylamine fragments were analyzed by SDS gel electrophoresis.

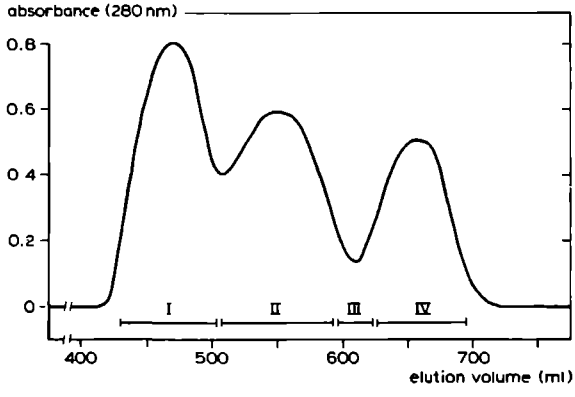

Fig. 20 Chromatography of the hydroxylamine fragments of reduced and S-alkylated LAP.
Approximately 300 mg of lyophilized HA fragments was applied to a column (200 x 3.5 cm) of Sephadex G-100 fine, and eluted with 10% (v/v) acetic acid containing 6 M urea at a flow rate of 10 ml/h. Fractions were pooled as indicated. Pool IV was further purified by ion-exchange chromatography (Fig. 21).

cleavage in the NH_2-terminal part of the polypeptide chain. Pool III yielded fragment HA 2 in pure form. The amino acid composition is listed in Table XXIII. The NH_2-terminal amino acid sequence of HA 2 was determined up to residue 27 by automated Edman degradation (Table XXIV).

Purification of CNBr fragments of reduced and S-aminoethylated hydroxylamine fragment 2. For the purification of CNBr fragments of HA 2 we used the incom-

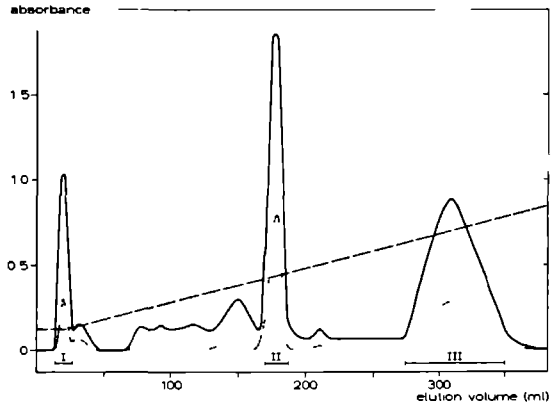

Fig. 21 Purification of fragment HA 2 of Ae-LAP by ion-exchange chromatography.
Pool IV of the G-100 column (Fig. 20) was applied to a column (8.0 x 2.0 cm) of Cm-cellulose in 0.01 M Na_2HPO_4, pH 5.8, containing 7 M urea, and eluted by means of a linear gradient made of 200 ml starting buffer (0.01 M Na_2HPO_4, pH 5.8) and 200 ml limit buffer (0.04 M Na_2HPO_4, pH 8.0) at a flow rate of 20 ml/h. Fractions were pooled as indicated.

pletely purified material of pool IV of the Sephadex G-100 column of Fig. 20, because further purification by ion-exchange chromatography gave too low yields of HA 2. The contaminating fragment in pool IV (Fig. 20), originating from the NH_2-terminal part of LAP, was not cleaved by CNBr and did not interfere with the purification of CNBr fragments of HA 2. The CNBr cleavage mixture of HA 2 was fractionated on Cm-cellulose in the presence of 7 M urea (Fig. 22), followed by gel filtration on Sephadex G-50 superfine and peptide mapping. Gel filtration of pool I and pool II of the Cm-cellulose

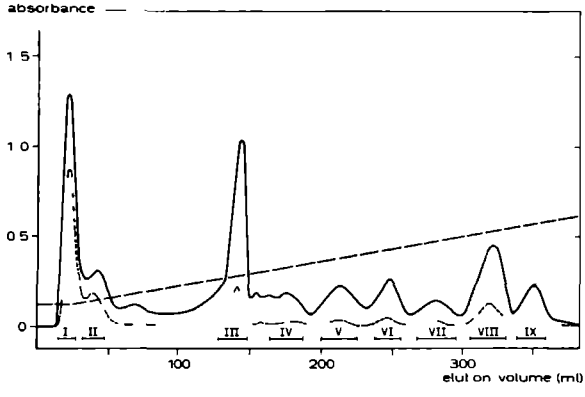

Fig. 22 Ion-exchange chromatography of the CNBr fragments of fragment HA 2 obtained from Ae-LAP.
Approximately 50 mg of lyophilized CNBr fragments was applied to a column (10.0 x 2.0 cm) of Cm-cellulose in 0.01 M Na_2HPO_4, pH 5.8, containing 7 M urea, and eluted by means of a linear gradient made of 200 ml starting buffer (0.01 M Na_2HPO_4, pH 5.8) and 200 ml limit buffer (0.03 M Na_2HPO_4, pH 8.0). Fractions were pooled as indicated. Pool I, II, III, VII and VIII were further purified by gel filtration, pool VI by peptide mapping.

column gave the same results; both pools contained two fragments, namely CB 9 and CB 10. Gel filtration of pool III (Fig. 22) is shown in Fig. 23. Pool III of the Sephadex G-50 superfine column (Fig. 23) yielded the NH_2-terminal CNBr

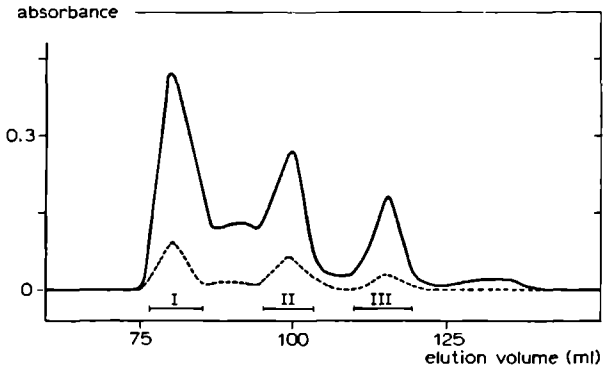

Fig. 23 Purification of CNBr fragments of fragment HA 2 obtained from Ae-LAP. Pool III of the Cm-cellulose column (Fig. 22) was applied to a column (110 x 1.6 cm) of Sephadex G-50 superfine in 20% (v/v) acetic acid and eluted with the same solvent at a flow rate of 6 ml/h. Fractions were pooled as indicated.

fragment of HA 2 in pure form (HA 2-CB 8b). The other pools contained no informative material. Pool IV and V of the Cm-cellulose column (Fig. 22) were also not informative. Peptide mapping of pool VI (Fig. 22) yielded fragment CB 12. Pool VII and VIII (Fig. 22) were analyzed by gel filtration on Sephadex G-50 superfine. In both cases only a single peak was obtained, containing fragment CB 11 in pure form. Tryptic digestion and peptide mapping of pool IX of the Cm-cellulose column (Fig. 22) showed that this pool contained the fragment resulting from incomplete cleavage between CB 11 and CB 12 at methionine 253 and some other impurities. In conclusion, fragment HA 2-CB 8b, CB 9, CB 10, CB 11 and CB 12 can be purified from fragment HA 2. The amino acid compositions of the purified fragments are included in Table III.

Sequence analysis of fragment HA 2-CB 8b. Fragment HA 2-CB 8b of Ae-LAP was investigated in order to complete the sequence of CB 8. Tryptic digestion followed by peptide mapping yielded peptides T 34b through T 37a (Table XXV). The amino acid composition of peptide T 36 is listed in Table XX. The amino acid compositions of the purified thermolytic peptides of HA 2-CB 8b are presented in Table XXVI. The accumulated evidence for this sequence of fragment HA 2-CB 8b is given in Fig. 24. Evidence for the overlap between T 36 and T 37a, which involved only one residue, was deduced from comparison of the amino acid composition of HA 2-CB 8b with the sum of the individual amino acids obtained from the sequence results (see Table III).

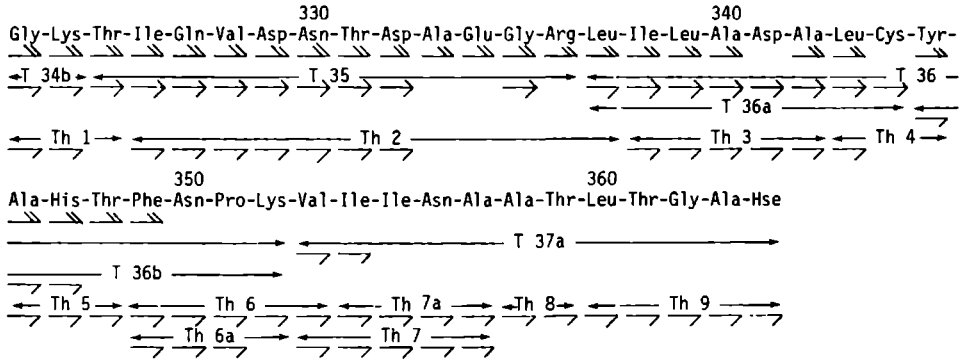

Fig. 24 Proposed amino acid sequence of CB 8b, the NH_2-terminal CNBr fragment of HA 2.
The residues 333-338 were obtained from a liquid phase sequencer run of fragment HA 2 (Table XXIV). The sequencer run also confirmed the alignment of the peptides T 34b to T 36.

Sequence analysis of fragment CB 11. The amino acid compositions of the tryptic peptides of CB 11 of Ae-LAP obtained from peptide mapping are shown in Table XXVII. Peptide T 41a could not be isolated in this way. The amino acid compositions of the peptides T 41 and T 43 were derived from the [^{3}H]-*S*-carboxymethylcysteine-containing tryptic peptides (see Fig. 18). Overlaps between the tryptic peptides were determined with the aid of chymotryptic, thermolytic and staphylococcal protease peptides of CB 11. The amino acid compositions of these peptides are presented in Tables XXVIII, XXIX and XXX, respectively. Sequence determination of the peptides and evidence for the alignment of the tryptic peptides are shown in Fig. 25. All overlaps between tryptic peptides involve at least two residues. The results of the sequence determination of CB 11 is in good agreement with the amino acid composition of this fragment.

Purification and sequence analysis of staphyloccoal protease peptide $SP^{232-304}$ of Ae-LAP. Because we did not succeed in the purification of the CNBr fragment representing residue 275-306, this part of the sequence was deduced from the fragment $SP^{232-304}$. Ae-LAP was digested with staphylococcal protease in the presence of 2 M urea. The digestion mixture was chromatographed on Sephadex G-75 fine (Fig. 26). Pool I of this elution pattern was rechromatographed on a Sephadex G-75 column (Fig. 27). Pool I of Fig. 27 contained fragment $SP^{232-304}$ in almost pure form. Fragment $SP^{232-304}$ was digested with trypsin and the resulting peptides were separated by peptide mapping. The amino acid compositions of the tryptic peptides are presented in Table XXXI. The se-

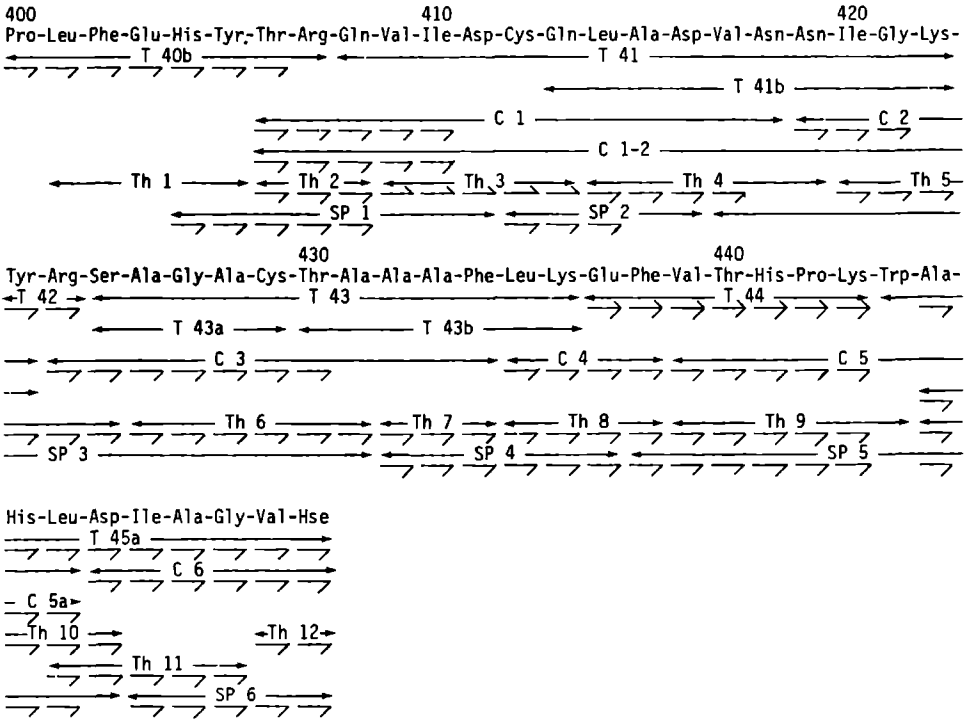

Fig. 25 Proposed amino acid sequence of fragment CB 11.
The amino acid sequence of the peptides T 41 and T 43 is presented in Fig. 18.

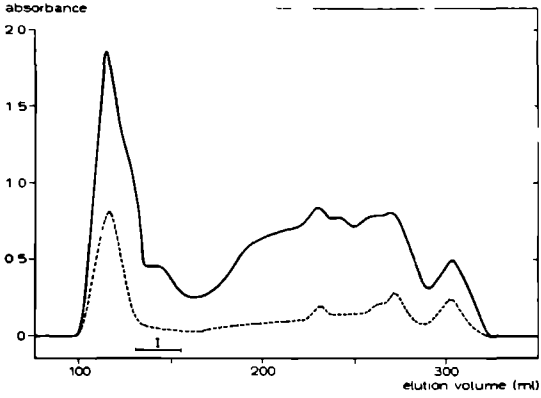

Fig. 26 Chromatography of a staphylococcal protease digest of Ae-LAP.
Approximately 50 mg of lyophilized digestion mixture was applied to a column (110 x 2.0 cm) of Sephadex G-75 fine and eluted with 0.1 M ammonia at a flow rate of 13 ml/h. Pool I was further purified by gel filtration (Fig. 27).

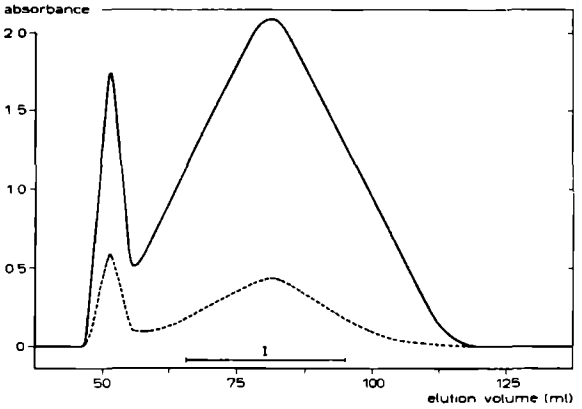

Fig. 27 Purification of the staphylococcal protease peptide SP$^{232-304}$. Pool I (Fig. 26) was applied to a column (110 x 1.3 cm) of Sephadex G-75 fine and eluted with 0.1 M ammonia at a flow rate of 9 ml/h. Pool I contained the fragment in almost pure form.

quence of T 30 and T 31 is described in Fig. 18 and is partially confirmed by the sequence analyses of T 30a, T 30b and T 31a (Fig. 28). Overlaps of the tryptic peptides were deduced from a chymotryptic digest of SP$^{232-304}$ (Table XXXII; Fig. 28). Overlap T 29-T 30a is confirmed by the sequence of CB 5 (res.

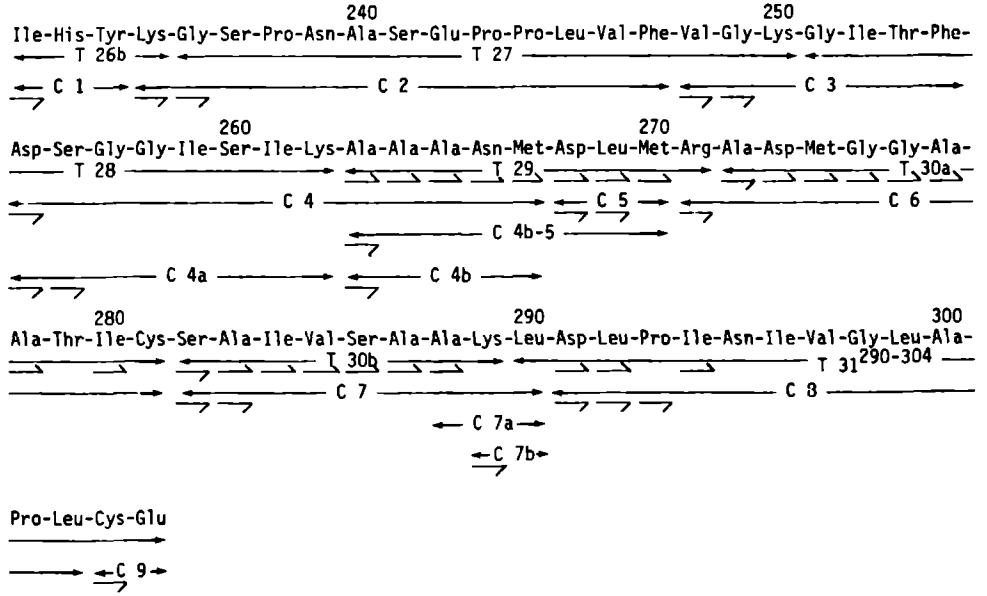

Fig. 28 Amino acid sequence of staphylococcal protease peptide SP$^{232-304}$. This sequence established the alignment of the CNBr fragments CB 4-CB 5-CB 6 and the sequence of the residues 275-304.

268-270) and CB 6 (res. 271-274). The overlap between T 30b-T 31a involved only one residue, but was confirmed by partial purification of tryptic pep-

tides from citraconylated LAP (results not shown). The overall sequence is presented in Fig. 28. The sequence of $SP^{232-304}$ established the alignment of the CNBr fragments CB 4-CB 5-CB 6 and the sequence of residues 275-304. The amino acid composition of $SP^{232-304}$ (Table XXXIII), which was indeed not completely pure, and therefore not in full agreement with the sequence determination, provided nevertheless evidence that no tryptic or CNBr peptides had been missed in this fragment.

Alignment of the CNBr fragments of HA 2 and complete sequence of this fragment. The accumulated evidence for the sequence of HA 2 is presented in Fig. 29*. Purification of a tryptic digest of HA 2 of Ae-LAP by peptide mapping yielded two methionine containing peptides T 40 and T 45 (Table XXXIV). Peptide T 45 contains a Lys-Asp bond which was not cleaved by trypsin. The partial sequences determined on these peptides are presented in Fig. 29*. The peptides obtained from a tryptic digest of citraconylated HA 2 of Ae-LAP were chromatographed on Sephadex G-50 superfine (Fig. 30). Fraction I, containing a mixture of the peptides HA 2-Tc 1 and HA 2-Tc 4, was subdigested by chymo-

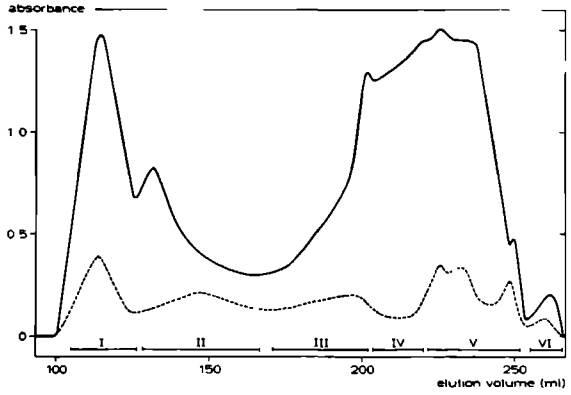

Fig. 30 Chromatography of the tryptic peptides of citraconylated HA 2 of Ae-LAP. Approximately 20 mg of the lyophilized digestion mixture was applied to a column (110 x 1.7 cm) of Sephadex G-50 superfine in 0.1 M ammonia and eluted with the same solvent at a flow rate of 6 ml/h. Fractions were pooled as indicated and decitraconylated.

trypsin (Table XXXV) and thermolysin (Table XXXVI). The NH_2-terminal residues of these peptides were determined by dansylation (Fig. 29*). Pool II (Fig. 30) also contained a mixture of HA 2-Tc 1 and HA 2-Tc-4. Pools III up to VI were analyzed by peptide mapping. The amino acid compositions of the purified peptides are shown in Table XXXVII and the sequence determination is given in Fig. 29*.

A chymotryptic digest of HA 2 of Cm-LAP was fractionated on a Sephadex G-

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*See page 64-65.

50 superfine column (Fig. 31). Pool I contained undigested HA 2. Pools II, III and IV were subjected to ion-exchange chromatography on DEAE-cellulose. Pools V up to VII were analyzed by peptide mapping. The amino acid analyses of the

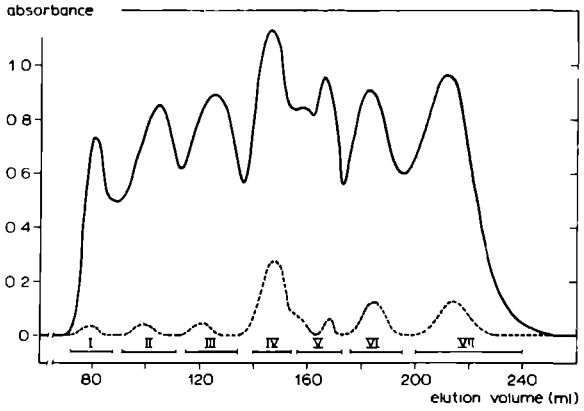

Fig. 31 Chromatography of the chymotryptic peptides of fragment HA 2 obtained from Cm-LAP.
A digestion mixture was applied to a column (110 x 1.7 cm) of Sephadex G-50 superfine and eluted with 0.1 M ammonia at a flow rate of 8 ml/h. Fractions were pooled as indicated. Pool II up to IV were further purified by ion-exchange chromatography, V up to VII by peptide mapping.

chymotryptic peptides are listed in Table XXXVIII and the sequence analysis is recorded in Fig. 29*. Some thermolytic peptides of HA 2 of Ae-LAP were isolated after performic acid oxidation and purified by peptide mapping. Two thermolytic peptides, which were Ehrlich positive, contained also methionine, HA 2-Th 2 and HA 2-Th 3 (Table XXXIX). Two other methionine-containing thermolytic peptides were purified by diagonal electrophoresis at pH 6.5; HA 2-Th1 and HA 2-Th4 (Table XXXIX). The partial sequence of the thermolytic peptides is presented in Fig. 29*. HA 2 of Ae-LAP was digested by staphylococcal protease in 2 M urea. After desalting over Sephadex G-10 the resulting peptide mixture was purified by peptide mapping. Five peptides were obtained (Table XL) and partially sequenced (Fig. 29*).

The sequence results providing evidence for the amino acid sequence of HA 2 are shown in Fig. 29*. The overlap between HA 2-CB 8b and HA 2-CB 9 (res. 364-365) is confirmed by peptides HA 2-C 3, HA 2-Tc 1-Th 3 and HA 2-Th 1. The alignment of CB 9 and CB 10 is supported by the amino acid composition and partial sequence of peptide HA 2-Th 2. The overlap is confirmed by subdigestion of peptide HA 2-Tc 1 with chymotrypsin (Table XXXV). The peptides HA 2-C7, HA 2-Th 3 and HA 2-SP 3 clearly establish the overlap between the fragments CB 10 and CB 11. The alignment CB 11-CB 12 is indicated by the amino acid composition and partial sequence of T 45, and further established by peptides HA 2-Tc 4-Th 8 and HA 2-Th 4. Further evidence for the sequence CB 11-CB 12 was obtained from the analysis of pool IX of the Cm-cellulose column

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*See page 64-65.

See opposite site:

Fig. 29 Proposed amino acid sequence of fragment HA 2.
The amino acid sequence was deduced by a combination of the results of CNBr fragments (Figs. 16, 24 and 25) and peptides establishing the alignment of CNBr fragments.

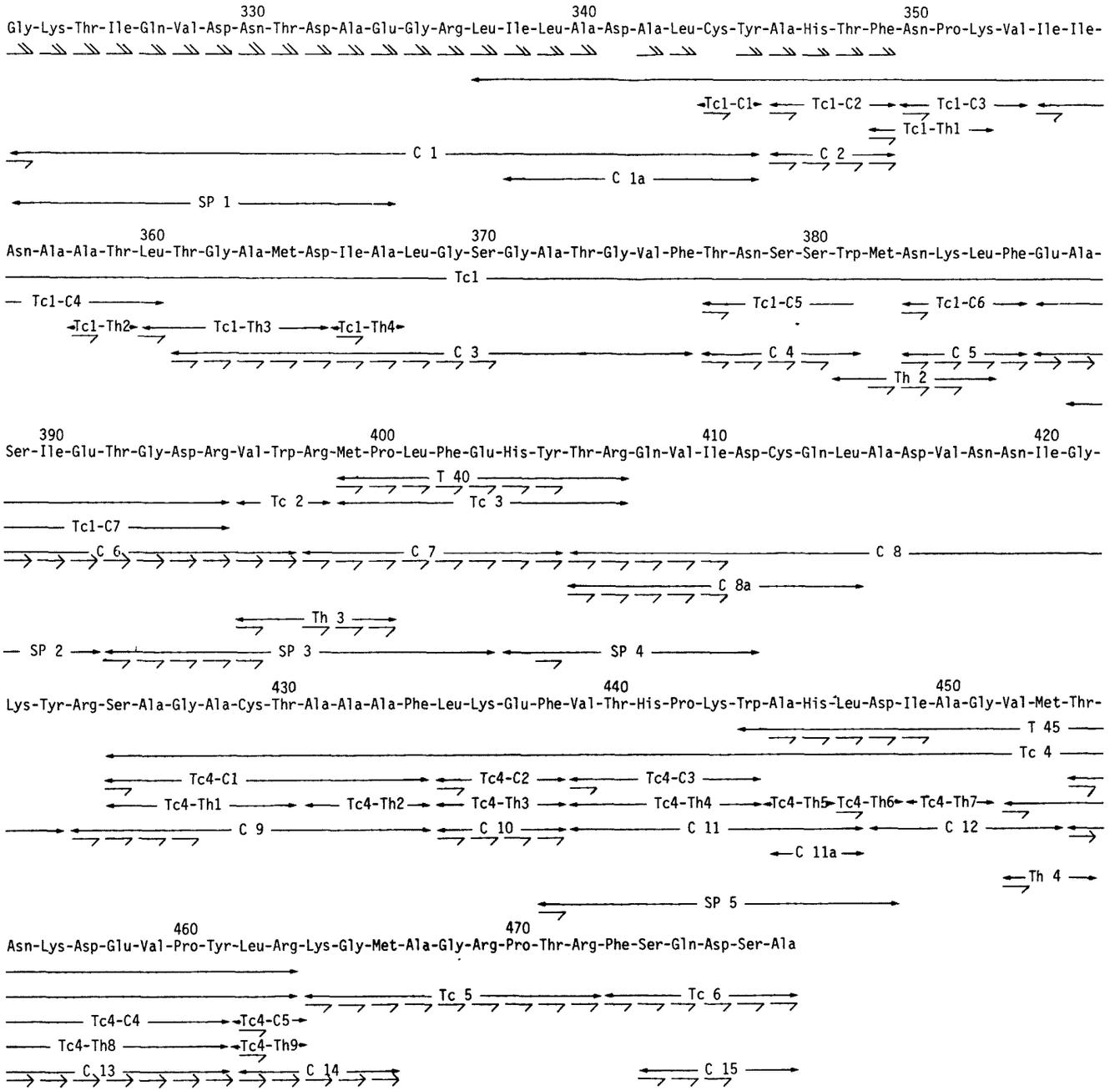
330
340
350
Gly-Lys-Thr-Ile-Gln-Val-Asp-Asn-Thr-Asp-Ala-Glu-Gly-Arg-Leu-Ile-Leu-Ala-Asp-Ala-Leu-Cys-Tyr-Ala-His-Thr-Phe-Asn-Pro-Lys-Val-Ile-Ile-
Tc1-C1
Tc1-C2
Tc1-C3
Tc1-Th1
C 1
C 2
C 1a
SP 1
360
370
380
Asn-Ala-Ala-Thr-Leu-Thr-Gly-Ala-Met-Asp-Ile-Ala-Leu-Gly-Ser-Gly-Ala-Thr-Gly-Val-Phe-Thr-Asn-Ser-Ser-Trp-Met-Asn-Lys-Leu-Phe-Glu-Ala-
Tc1
Tc1-C4
Tc1-C5
Tc1-C6
Tc1-Th2
Tc1-Th3
Tc1-Th4
C 3
C 4
C 5
Th 2
390
400
410
420
Ser-Ile-Glu-Thr-Gly-Asp-Arg-Val-Trp-Arg-Met-Pro-Leu-Phe-Glu-His-Tyr-Thr-Arg-Gln-Val-Ile-Asp-Cys-Gln-Leu-Ala-Asp-Val-Asn-Asn-Ile-Gly-
T 40
Tc 2
Tc 3
Tc1-C7
C 6
C 7
C 8
C 8a
Th 3
SP 2
SP 3
SP 4
430
440
450
Lys-Tyr-Arg-Ser-Ala-Gly-Ala-Cys-Thr-Ala-Ala-Ala-Phe-Leu-Lys-Glu-Phe-Val-Thr-His-Pro-Lys-Trp-Ala-His-Leu-Asp-Ile-Ala-Gly-Val-Met-Thr-
T 45
Tc 4
Tc4-C1
Tc4-C2
Tc4-C3
Tc4-Th1
Tc4-Th2
Tc4-Th3
Tc4-Th4
Tc4-Th5
Tc4-Th6
Tc4-Th7
C 9
C 10
C 11
C 12
C 11a
Th 4
SP 5
460
470
Asn-Lys-Asp-Glu-Val-Pro-Tyr-Leu-Arg-Lys-Gly-Met-Ala-Gly-Arg-Pro-Thr-Arg-Phe-Ser-Gln-Asp-Ser-Ala
Tc 5
Tc 6
Tc4-C4
Tc4-C5
Tc4-Th8
Tc4-Th9
C 13
C 14
C 15

Fig. 22, which contained the incomplete cleavage product CB 11-12. The CNBr fragment representing residues 467-478 was never purified. The sequence 467-478 was deduced from the peptides HA 2-Tc 5 and HA 2-Tc 6. Peptide HA 2-Tc 5 also determined the sequence around Met-466. The order of HA 2-Tc 5 and HA 2-Tc 6 was only based on the fact, that the residue preceeding peptide HA 2-Tc 6 should be an arginine, and because comparison of the amino acid composition of HA 2 with the number of residues of the basic amino acids lysine and arginine obtained from the sequence determination (Table XXIII) indicate that no citraconyl peptide was lost. We consider peptide HA 2-Tc 6, which terminates with alanine, as the COOH-terminal peptide of HA 2 and hence of the total polypeptide chain of LAP. This peptide was also purified from a tryptic peptide map of HA 2 and from a tryptic peptide mixture of citraconylated LAP (results not shown).

Purification of peptides establishing the overlap of CB-fragments. In order to establish the overlap CB 1-CB 2, a peptide (res. 170-205) was isolated from a tryptic digest of citraconylated Ae-LAP. The digestion mixture was chromatographed on G-75 (Fig. 32). Pool I was subsequently subjected to ion-exchange chromatography on a DEAE-cellulose column (Fig. 33) yielding a pure fragment

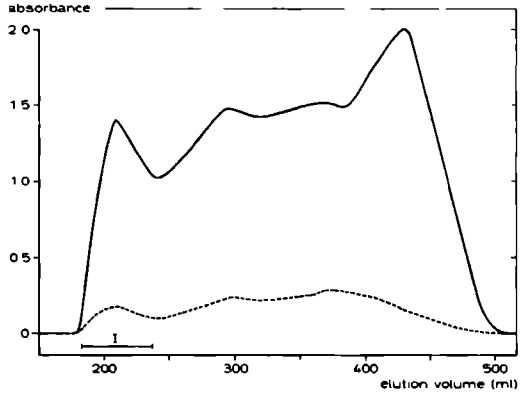

Fig. 32 Fractionation of the tryptic peptides of citraconylated Ae-LAP.
Approximately 80 mg of the citraconylated digestion mixture was applied to a column (140 x 2.0 cm) of Sephadex G-75 fine eluted with 0.1 M ammonia at a flow rate of 10 ml/h. Pool I was decitraconylated and subjected to ion-exchange chromatography (Fig. 33).

$Tc^{170-205}$ (pool I). This fragment was digested with trypsin and with staphylococcal protease. The purified tryptic peptides T 22 to T 24 are listed in Table XLI. Staphylococcal protease digestion yielded six peptides of which the amino acid analyses are presented in Table XLI. Peptide $Tc^{170-205}$-SP 1 was subjected to four cycles of Edman degradation. The sequence established the overlap CB 1-CB 2 (Fig. 34). This citraconyl peptide had COOH-terminal

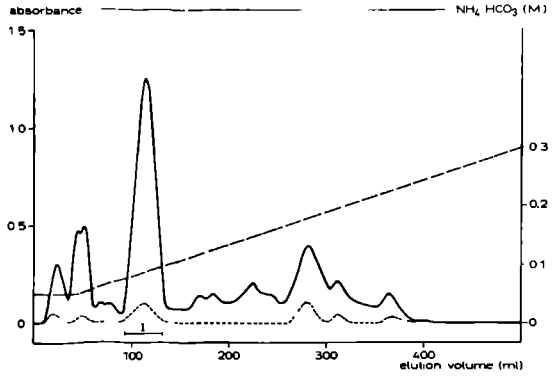

Fig. 33 Purification of peptide $Tc^{170-205}$ by ion-exchange chromatography.
Pool I of the Sephadex G-75 column (Fig. 32) was dissolved in 0.05 M NH_4HCO_3, pH 7.8, and brought onto a column (10.0 x 1.0 cm) of DEAE-cellulose (DE-52) equilibrated in the same buffer. Elution was performed with a linear gradient made of 250 ml starting buffer and 250 ml of limit buffer (0.3 M NH_4HCO_3, pH 7.8) at a flow rate of 20 ml/h. Pool I contained the peptide in almost pure form.

lysine, probably because Pro-204 hindered the blocking of Lys-205 by citraconylation.

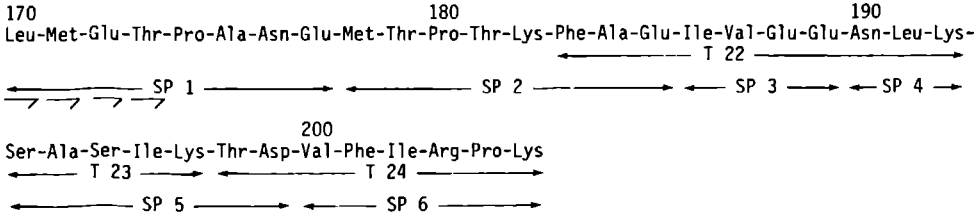

Fig. 34 Amino acid sequence determination of peptide $Tc^{170-205}$ of citraconylated LAP.
This sequence established the order CB 1-CB 2.

The overlap CB 2-CB 3 was established by the purification of peptide $T^{172-182}$. Tryptic digestion and peptide mapping of pool II (Fig. 8), which contained a mixture of two fragments (CB 2-3 and CB 4) yielded peptide $T^{172-182}$, together with the tryptic peptides of CB 3 and CB 4. The amino acid composition is shown in Table XLII and the sequence determination up to residue 9 in Fig. 35. The sequence confirms the overlap between the fragments CB 2 and CB 3 (Fig. 4).

To confirm the overlap between CB 3 and CB 4 peptide T 25 was isolated. Analysis of pool II (Fig. 17) by peptide mapping yielded one negatively charged peptide (T 25) which contained tryptophan, as determined by Ehrlich's staining. The amino acid composition is presented in Table XLII and the amino acid sequence, determined up to the penultimate residue, in Fig. 35. The sequence of T 25 established the alignment CB 3-CB 4 (Fig. 4). This overlap is

also confirmed by the tryptic map of pool I in Fig. 7. This fraction contained an almost pure fragment, that resulted from the incomplete cleavage between CB 3 and CB 4 at Met-213. Peptide T 25 was again purified, and yielded the same amino acid composition as shown in Table XLII, except that homoserine replaced methionine (results not shown).

Peptide	Sequence
$T^{172-182}$	172 180 Glu-Thr-Pro-Ala-Asn-Glu-Hse-Thr-Pro-Thr-Lys
T 25	210 220 Ser-Trp-Ile-Glu-Glu-Gln-Glu-Met-Gly-Ser-Phe-Leu-Ser-Val-Ala-Lys

Fig. 35 Amino acid sequence determination of peptides establishing the overlap of CNBr-fragments.

The alignment of the fragments CB 4-CB 5-CB 6 is confirmed by the sequence of $SP^{232-304}$ (Fig. 28). The amino acid sequence of peptide T 31 (Fig. 18) provided the overlap between $SP^{232-304}$ and CB 8. The remaining overlaps are established by the amino acid sequence of fragment HA 2 (Fig. 29).

Table III. Amino acid composition of the cyanogen bromide fragments purified from reduced and S-alkylated leucine aminopeptidase and from hydroxylamine fragment 2. Values are given as the number of residues per fragment as determined by amino acid analysis. Values between brackets are the actual values found in the sequence. Values for valine and isoleucine were taken from the 72 h hydrolysate and values for threonine and serine were extrapolated to zero time hydrolysis.

	Fragments					
	CB 1	CB 2	CB 3	CB 4	CB 5	CB 6
Asp	14.8 (15)	1.2 (1)	2.2 (2)	3.2 (3)	1.0 (1)	1.0 (1)
Thr	5.2 (5)	1.1 (1)	3.0 (3)	0.9 (1)		
Ser	8.4 (9)		2.9 (3)	6.7 (7)		
Hse	0.7 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Glu	25.0 (26)	1.9 (2)	7.0 (7)	4.0 (4)		
Pro	6.3 (6)	1.0 (1)	2.5 (2)	5.2 (5)		
Gly	17.7 (18)			7.0 (7)		
Ala	17.0 (16)	0.9 (1)	2.1 (2)	5.1 (5)		1.0 (1)
Cys	1.8[a] (2)					
Val	13.4 (14)		2.0 (2)	4.1 (4)		
Ile	7.1 (7)		3.7 (4)	4.0 (4)		
Leu	15.7 (16)		1.2 (1)	3.2 (3)	1.0 (1)	
Tyr	3.7 (4)			1.0 (1)		
Phe	5.3 (5)		1.8 (2)	4.2 (4)		
His	3.2 (3)			1.0 (1)		
Lys	13.8 (14)		3.7 (4)	4.2 (4)		
Arg	8.0 (8)		1.0 (1)			0.9 (1)
Trp	n.d (2)		n.d (1)			
Total	171	7	35	54	3	4

	Fragments				
	CB 8b[b,c,d]	CB 9[c]	CB 10[c]	CB 11[c]	CB 12[c]
Asp	6.1 (6)	2.1 (2)	1.0 (2)	5.2 (5)	2.0 (2)
Thr	5.1 (5)	1.9 (2)	0.9 (1)	3.1 (3)	0.8 (1)
Ser		2.8 (3)	0.9 (1)	1.2 (1)	
Hse	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Glu	2.4 (2)		2.0 (2)	4.0 (4)	1.1 (1)
Pro	0.9 (1)			2.0 (2)	1.1 (1)
Gly	3.1 (3)	3.0 (3)	1.1 (1)	2.9 (3)	1.2 (1)
Ala	6.9[b] (7)	2.1 (2)	1.1 (1)	8.0[b] (8)	
Cys	1.0[b] (1)			1.6[b] (2)	
Val	2.0 (2)	0.9 (1)	0.9 (1)	3.6 (4)	1.2 (1)
Ile	3.8 (4)	1.1 (1)	1.0 (1)	2.7 (3)	
Leu	4.1 (4)	1.1 (1)	1.3 (1)	3.9 (4)	1.0 (1)
Tyr	0.9 (1)			1.8 (2)	1.0 (1)
Phe	1.0 (1)	1.0 (1)	1.0 (1)	3.7 (3)	
His	1.0 (1)			3.0 (3)	
Lys	2.2 (2)		1.1 (1)	3.0 (3)	2.0 (2)
Arg	1.2 (1)		2.0 (2)	2.4 (2)	1.1 (1)
Trp		n.d.(1)	n.d.(1)	n.d (1)	
Total	42	18	17	54	13

n.d. = not determined

[a] determined as S-carboxymethylcysteine
[b] determined as S-aminoethylcysteine
[c] purified from HA 2
[d] CB 8b is the first cyanogen bromide fragment of HA 2

Table II. Automatic Edman degradation of bovine lens leucine aminopeptidase. Approximately 0.5 µmol of sample was subjected to automatic Edman degradation in a Beckman sequencer. Residues were identified as phenylthiohydantoin by thin-layer chromatography and quantified by gas chromatography. The repetitive yield was 94 %.

Cycle	Residue	Yield (nmol)
1	Thr[a]	
2	Lys	
3	Gly	350
4	Leu	345
5	Val	320
6	Leu	300
7	Gly	280
8	Ile	250
9	Tyr	
10	Ser	
11	Lys	
12	Glu	200
13	Lys	
14	Glu	190
15	Glu	180
16	Asp	150
17	Glu	120
18	Pro	100
19	Gln	
20	Phe	80
21	Thr	

[a] The NH_2-terminal threonine was also identified by dansylation

Table IV Amino acid compositions of the tryptic peptides of CB 1
Peptides were isolated by gel filtration on Sephadex G-50 (A), by peptide mapping (B), after re-electrophoresis at pH 3 5 (C), and by ion-exchange chromatography on DEAE-cellulose (D) Peptide T 2 was not isolated

	Peptides									
	T 1	T 2	T 3	T 4	T 5	T 6	T 7	T 8	T 9	T 10
Asp			2 9(3)			0 9(1)			0.9(1)	
Thr	0 9(1)		1 0(1)					1 0(1)	1.0(1)	
Ser		(1)	0 9(1)	0 9(1)		0 9(1)			1 0(1)	
Glu			5.6(6)			1 1(1)			1 0(1)	
Pro			1 2(1)			1 9(2)			1.0(1)	
Gly		(2)	1 0(1)	1 2(1)		1 2(1)	1 0(1)		3.2(3)	
Ala			1 0(1)				1 0(1)			
Val		(1)		1 0(1)					3 2(4)[a]	
Ile		(1)				1 8(2)				
Leu		(2)		0 9(1)	1.0(1)	2.0(2)			2.3(2)	
Tyr		(1)							0 9(1)	
Phe			1 9(2)						1 9(2)	
His									1 0(1)	
Lys	1 0(1)	(1)	1 9(2)	1 0(1)		1 0(1)	1.0(1)		1.0(1)	1 0(1)
Arg					1.0(1)			1 0(1)		
Total	2	(9)	18	5	2	11	3	2	19	1
Yield(%)	25	-	38	36	40	50	25	26	10	10
Purif	AB		AB	AB	AB	AB	AB	AB	AB	AB
Charge	+1		-3	+1	+1	0	+1	+1	0	+1

	Peptides									
	T 10-11	T 11	T 12	T 13	T 14	T 14a	T 14b	T 15	T16	T 16-17
Asp	1 9(2)	1 8(2)	1 0(1)		4.5(5)	2 1(2)	2 7(3)			
Thr	0 9(1)	1.0(1)								
Ser					0 7(1)	0 9(1)				0 9(1)
Glu	4 0(4)	3 8(4)	1.0(1)		6.8(7)	4.2(4)	3.0(3)	1.0(1)		
Pro					1.9(2)	2.0(2)				
Gly	2 2(2)	2.0(2)		1 1(1)	2.1(3)		2 5(3)			
Ala	1 1(1)	1 2(1)		3.9(4)	4 6(5)		4 9(5)			1 0(1)
Cys				1.0(-)[b]	0.8(1)[b]	0 9(1)[c]				
Val				1.0(1)	2.5(3)	1.8(2)	0.9(1)			2.4(3)[a]
Ile	1.0(1)	1 1(1)	1 0(1)		1 6(2)	1.9(2)				
Leu					3.7(4)	1 1(1)	2 2(3)			
Tyr					1.8(2)		1 5(2)			
His	1 0(1)	1 0(1)								
Lys	2 0(2)	1 0(1)			1.0(1)		0 9(1)	1 0(1)	1.0(1)	2.0(2)
Arg			1 0(1)	1.1(1)				0 9(1)		
Trp	+ (1)	+ (1)								
Total	15	14	4	8	36	15	21	3	1	7
Yield( )	8	30	42	23	75	8	6	11	18	13
Purif	AB	AB	ABC	ABC	A	AB	AB	AB	AB	AB
Charge	0/-1	-1	0	0		-7/-3	-3	+2	+1	+2

[a]Value taken from the 72 h hydrolysate
[b]Determined as S-carboxymethylcysteine
[c]Determined as S-aminoethylcysteine

Table IV. (cont'd)

	Peptides						
	T 17	T 18	T 19	T 20	T 20-21	T 21	Total
Asp		1.0(1)	0 9(1)				15
Thr							5
Ser	0 8(1)	0 9(1)	0 9(1)				9
Hse					0 7(1)	0 6(1)	1
Glu		3.6(4)	1 1(1)				26
Pro							6
Gly		0.9(1)	1 9(2)				18
Ala	1.0(1)	0 9(1)	2 1(2)				16
Cys							2
Val	2.3(3)[a]		0 9(1)				14
Ile							7
Leu		1.0(1)	2 0(2)		1 0(1)	1 0(1)	16
Tyr							4
Phe			1.0(1)				5
His		1 0(1)					3
Lys	1.1(1)						14
Arg		1.1(1)	1 0(1)	1 0(1)	1.0(1)		8
Trp		+ (1)					2
Total	6	12	12	1	3	2	171
Yield(%)	24	39	33	5	15	17	
Purif.	AB	AB	AB	AB	AB	AB	
Charge	+1	-1	+1	+1	+1	0	

[a]Value taken from the 72 h hydrolysate

Table VI. Amino acid compositions of the chymotryptic peptides of CB 1.
All peptides were purified by a combination of gel filtration and peptide mapping, except peptide C 14 which was already pure after the gel filtration procedure.

	Peptides										
	C 1	C 1-2	C 2-3	C 3	C 4	C 5	C 6	C 7	C 8	C 9	C 10
Asp					1.9(2)	1.0(1)		1.0(1)			0.9(1)
Thr	1.0(1)	0.8(1)			1.0(1)				1.9(2)		
Ser					1.7(2)		1.0(1)	1.0(1)			0.9(1)
Glu					6.0(6)		1.0(1)				1.1(1)
Pro					0.9(1)			2.1(2)			1.0(1)
Gly	0.9(1)	1.0(1)	1.2(1)	1.0(1)	0.9(1)		1.2(1)	1.1(1)	1.1(1)		2.0(2)
Ala					1.0(1)				1.2(1)		
Val		1.1(1)	1.0(1)				1.0(1)				3.5(4)[b]
Ile			0.9(1)	1.0(1)			1.0(1)	1.0(1)			
Leu	1.1(1)	1.9(2)	1.0(1)			1.0(1)	1.9(2)	1.2(1)			1.9(2)
Tyr			0.9(1)	0.9(1)						1.0(1)	
Phe					2.0(2)				1.1(1)		1.1(1)
His											1.2(1)
Lys	1.0(1)	1.0(1)			1.8(2)	1.0(1)	1.2(1)		1.9(2)		
Arg							1.0(1)		1.0(1)		
Total	4	6	5	3	18	3	9	7	8	1	14
Yield(%)	22	11	19	15	35	31	20	11	8	12	26
Charge	+1	+1	0	0	-3	+1	+1	0	+3	0	-1

	Peptides										
	C 11	C 11-12	C 12-13	C 14	C 15	C 16	C 17	C 18	C 19	C 20	C 21
Asp	1.9(2)	2.0(2)	1.0(1)	3.0(3)		1.9(2)		1.2(1)		1.0(1)	
Thr	1.1(1)	1.1(1)									
Ser				0.6(1)			0.9(1)	0.9(1)		0.9(1)	
Hse											0.8(1)
Glu	3.1(3)	3.0(3)	2.0(2)	5.6(6)		0.9(1)		2.9(3)	1.0(1)	1.2(1)	
Pro				1.8(2)							
Gly	2.0(2)	1.9(2)	1.2(1)	2.6(3)	1.0(1)			1.1(1)	1.1(1)	1.0(1)	
Ala	1.2(1)	1.2(1)	2.1(2)	6.3(7)			1.0(1)	1.1(1)		1.1(1)	1.1(1)
Cys				1.8(2)[a]							
Val			1.0(1)	2.7(3)			2.6(3)[b]		0.9(1)		
Ile	1.0(1)	1.1(1)	0.9(1)	1.8(2)							
Leu				1.9(2)	0.8(1)	1.0(1)	1.0(1)		1.0(1)	0.9(1)	1.0(1)
Tyr					1.0(1)	1.0(1)					
Phe									1.0(1)		
His		1.0(1)	0.9(1)					0.9(1)			
Lys	1.8(2)	2.0(2)	1.1(1)				1.0(1)				
Arg			1.1(1)	1.0(1)					1.0(1)		2.0(2)
Trp	+ (1)	+ (1)						+ (1)			
Total	13	14	11	32	3	5	7	9	6	6	5
Yield(%)	8	14	13	80	35	8	25	17	17	22	8
Charge	-1	0	0		0	-3	+1	-2	+1	0	+2

[a]Determined as S-carboxymethylcysteine
[b]Value taken from the 72 h hydrolysate

Table V. Amino acid compositions of peptides obtained by thermolytic (Th) or chymotryptic (C) digestion of some tryptic peptides of CB 1.
All peptides were purified by peptide mapping.

	Peptides									
	T 3-C 1	T 3-C 2	T 3-C 3	T 3-Th	T 6-C 1	T 6-C 2	T 9-Th1	T 9-Th2	T 9-Th3	T 9-Th4
Asp	1.2(1)	1.3(1)	1.0(1)	1.0(1)		0.9(1)		1.1(1)		
Thr		0.9(1)					0.9(1)			
Ser		0.9(1)				0.7(1)		0.9(1)		
Glu	4.6(5)	1.4(1)			0.8(1)			1.0(1)		
Pro	1.0(1)					1.8(2)		0.7(1)		
Gly		0.8(1)				1.0(1)	1.0(1)		1.0(1)	1.1(1)
Ala		0.8(1)								
Val									3.6(4)[a]	
Ile					1.0(1)	1.0(1)				
Leu					1.0(1)	1.0(1)		0.9(1)		1.0(1)
Tyr							0.8(1)			
Phe	0.9(1)	1.0(1)		1.0(1)			1.0(1)	1.0(1)		
His								0.9(1)		
Lys	1.0(1)		1.0(1)	1.0(1)		1.2(1)				1.0(1)
Charge	-3	-1	+1	+1	-1	+1	0	-1	0	+1

	Peptides								
	T 11-Th1	T 11-Th2	T 12-Th1	T 12-Th2	T 18-C 1	T 18-C 2	T 19-C 1	T 19-C 2	T 19-C 3
Asp		1.9(2)	1.0(1)		1.0(1)			1.0(1)	
Thr	0.8(1)								
Ser					0.9(1)			0.9(1)	
Glu		3.9(4)	0.9(1)		2.8(3)	0.9(1)		1.0(1)	
Gly	1.9(1)	1.2(1)			1.0(1)		1.0(1)	1.0(1)	
Ala	1.0(1)				1.0(1)			1.0(1)	0.9(1)
Val							0.9(1)		
Ile		1.1(1)		0.9(1)					
Leu					1.0(1)		0.9(1)	1.1(1)	
Phe							1.0(1)		
His		1.0(1)			0.9(1)				
Lys		1.0(1)							
Arg				1.1(1)		1.0(1)			1.0(1)
Trp		+ (1)			+ (1)				
Charge	0	-2	-1	+1	-2	+1	0	0	+1

[a]Value taken from 72 h hydrolysate

Table VII Amino acid composition of the thermolytic peptides of CB 1
All peptides were purified by peptide mapping

	Peptides									
	Th 1	Th 2	Th 3	Th 4	Th 5	Th 5-6	Th 6-7	Th 7	Th 8	Th 9
Asp			1 2(1)	1 2(1)	1 0(1)	1 0(1)				1 1(1)
Thr	0 9(1)			0 9(1)						
Ser			1 0(1)	1 0(1)			0 9(1)	0 8(1)		
Glu			5 2(5)	1 1(1)					1 0(1)	
Pro			1 0(1)							
Gly	1 1(1)	1 1(1)		1 1(1)			1 0(1)	1 1(1)		
Ala				1 0(1)						
Val		0 7(1)					0 9(1)	0 9(1)		
Ile			0 7(1)							0 9(1)
Leu		2 0(2)				1 0(1)	1 0(1)		1 1(1)	1 1(1)
Tyr			0 9(1)							
Phe				1 0(1)	0 9(1)	0 9(1)				
Lys	1 0(1)		1 9(2)		1 1(1)	1 0(1)	1 0(1)	1 0(1)		
Arg									0 9(1)	
Total	3	4	12	7	3	4	5	4	3	3
Yield( )	15	19	8	17	21	7	18	11	14	15
Charge	+1	0	-3	-1	+1	+1	+1	+1	0	0

	Peptides									
	Th 10	Th 10-11	Th 12	Th 13	Th 14	Th 15	Th 16	Th 16-17	Th 18	Th 19
Asp				1 1(1)				3 1(3)		
Thr		2 0(2)				1 0(1)				
Ser	1 1(1)	0 9(1)		0 9(1)						
Glu				1 1(1)				4 8(5)		0 8(1)
Pro	2 0(2)	1 9(2)		0 9(1)						
Gly	1 0(1)	2 0(2)	1 0(1)		0 9(1)	1 0(1)	1 0(1)	2 0(2)		1 2(1)
Ala		1 0(1)					1 0(1)	1 1(1)	1 9(2)	2 0(2)
Cys										0 9(1)[b]
Val					3 5(4)[a]					1 0(1)
Ile	1 0(1)	0 9(1)						1 0(1)	1 0(1)	
Leu		1 1(1)		1 0(1)		1 2(1)				
Tyr			0 9(1)							
Phe			0 9(1)	1 0(1)						
His				1 0(1)				1 0(1)		
Lys		1 9(2)				2 1(2)		1 0(1)		
Arg		0 9(1)							1 0(1)	1 0(1)
Trp								+ (1)		
Total	5	13	3	7	5	5	2	15	4	7
Yield(%)	14	10	17	17	17	15	15	19	13	18
Charge	0	+3	0	1	0	+2	0	-3	+1	0

[a] Value taken from the 72 h hydrolysate

[b] Determined as S carboxymethylcysteine

Table VII (cont'd)

	Peptides							
	Th 20	Th 20-21	Th 22	Th 23	Th 24	Th 25	Th 26	Th 27
Asp	1 1(1)	2 9(3)	2 1(2)		1 0(1)	1 1(1)		
Ser		0 8(1)		0 9(1)	0 9(1)	1 0(1)		
Hse								0 8(1)
Glu	1 0(1)	4 7(5)	1 8(2)		4 1(4)	1 2(1)		
Pro		1 9(2)						
Gly		1 7(2)	1 0(1)		2 0(2)	1 0(1)		
Ala		4 6(5)[b]		1 2(1)	1 1(1)	1 0(1)	1 0(1)	
Cys		0 8(1)[a]						
Val		2 8(3)[a]		2 6(3)[a]				
Ile	0 9(1)	1 7(2)						
Leu		0 7(1)	2 8(3)		0 8(1)		0 9(1)	1 0(1)
Tyr			2 1(2)					
Phe						1 0(1)		
His					0 9(1)			
Lys			3 1(3)	0 9(1)				
Arg			0 9(1)		0 9(1)		1 9(2)	
Trp					+ (1)			
Total	3	25	14	6	13	6	4	2
Yield(%)	6	19	13	12	9	18	15	19
Charge	-1	-3	+1	+1	-1	0	+2	0

[a] Value taken from the 72 h hydrolysate

[b] Determined as S-carboxymethylcysteine

Table X Amino acid composition of the thermolytic peptides of CB 3. Peptides were isolated by peptide mapping.

	Peptides							
	Th 1	Th 2	Th 3	Th 4	Th 5a	Th 5	Th 6	Th 7
Asp			0.9(1)			1.1(1)		
Thr	1.9(2)					1.0(1)		
Ser				0.9(1)			1.0(1)	
Hse								1 0(1)
Glu		1.0(1)	2.1(2)					5.0(4)
Pro	1.0(1)						0.9(1)	
Ala		1.0(1)						
Val			0.9(1)[a]		1.0(1)	0 9(1)		
Ile			0.9(1)[a]			0.9(1)	0.9(1)	1.0(1)
Leu				1.0(1)				
Phe		1.0(1)			1.0(1)	1.0(1)		
Lys	1.1(1)			1 1(1)		1.1(1)	1 2(1)	
Arg							1 0(1)	
Trp							+ (1)	
Total	4	3	5	3	2	6	6	6
Yield(%)	20	29	27	32	15	13	7	18
Charge	+1	-1	-2	+1	0	0	+2	-2/-3

[a]Value taken from the 72 h hydrolysate

Table XI. Amino acid composition of the tryptic peptides of CB 4 Peptides were purified by peptide mapping.

	Peptides				
	T 25b	T 26	T 27[a]	T 28	T 29a
Asp			1.0(1)	1.0(1)	1 1(1)
Thr				1.0(1)	
Ser	1.8(2)	0.9(1)	1.7(2)	1.8(2)	
Hse					1.0(1)
Glu		3 2(3)	1.3(1)		
Pro		1 8(2)	3.1(3)		
Gly	1.2(1)	0.9(1)	1.7(2)	3.2(3)	
Ala	1.2(1)		1.1(1)		?.9(3)
Val	1.1(1)	1 1(1)	2 1(2)		
Ile		0 9(1)		2 9(3)	
Leu	1.1(1)	1 1(1)	1.0(1)		
Tyr		1.0(1)			
Phe	1.2(1)	1.0(1)	1.0(1)	1 1(1)	
His		1 0(1)			
Lys	1.0(1)	1.0(1)	1.0(1)	1.0(1)	
Total	8	14	15	12	5
Yield(%)	21	14	5	20	18
Charge	+1	-1	0	0	0

[a]T 27 was partially modified by deamidation of residue 239 during purification

Table VIII. Amino acid composition of the tryptic peptides of citraconylated CB 1. Peptides were isolated by gel filtration (A) followed by ion-exchange chromatography (B) or peptide mapping (C). Values between brackets are the actual values found in the sequence

	Peptides								
	Tc 1	Tc 2	Tc 3	Tc 4	Tc 5	Tc 6	Tc 7	Tc 8	Tc 9
Asp	2 9(3)	1.0(1)	3.9(4)		5 1(5)	1 1(1)	1.1(1)		
Thr	1 7(2)	1 0(1)	2 1(2)						
Ser	2 8(3)	0 9(1)	1.2(1)		1.2(1)	1.8(2)	0.8(1)		
Hse									0 9(1)
Glu	6 0(6)	0 8(1)	6 0(6)		7 5(8)	3.7(4)	1.0(1)		
Pro	0 8(1)	1.8(2)	1 0(1)		2 0(2)				
Gly	4.0(4)	2.0(2)	5.0(5)	1 1(1)	2 8(3)	1 0(1)	1.7(2)		
Ala	0.9(1)	1.2(1)	1 3(1)	4 1(4)	4 6(5)	1 9(2)	2.1(2)		
Cys				1 0(1)[a]	0 9(1)[a]				
Val	1 9(2)		3 8(4)[b]	1.0(1)	2 3(3)	2.7(3)[b]	1 0(1)		
Ile	1.0(1)	1.9(2)	1.8(2)		2.0(2)				
Leu	3.9(4)	2.0(2)	2.3(2)		3.8(4)	1.0(1)	2.0(2)		1 0(1)
Tyr	1.0(1)		1 2(1)		1.8(2)				
Phe	2.0(2)		2 0(2)				1.1(1)		
His			1.9(2)			1.0(1)			
Lys	4 9(5)	1 7(2)	3.0(3)		2.0(2)	2.0(2)			
Arg	1 0(1)	0 9(1)	1 0(1)	1 0(1)	1.0(1)	1.0(1)	1 0(1)	1.0(1)	
Trp			(1)			(1)			
Total	36	16	38	8	39	19	12	1	2
NH_2-term	Thr	Glx	Thr	Ala	Glx	Lys	Gly	Arg	Leu
Purif	AB	AB	AB	AC	AB	AB	AC	AC	AC

[a]Determined as S-carboxymethylcysteine

[b]Value taken from the 72 h hydrolysate

Table IX. Amino acid composition of the tryptic and chymotryptic peptides of CB 3. All peptides were purified by peptide mapping.

	Peptides						
	T 21c	T 22	T 23	T 24	T 25a	C 1	C 2
Asp		1.0(1)		1 0(1)			
Thr	1.7(2)			0.8(1)		1 6(2)	
Ser			1.8(2)		0 7(1)		0.8(1)
Hse					1.0(1)		
Glu		2.9(3)			4.3(4)		
Pro	1.1(1)			1 1(1)		0.7(1)	1.0(1)
Ala		1.0(1)	1.2(1)				
Val		0.8(1)		1.1(1)			
Ile		1.0(1)	1.1(1)	0.9(1)	1.0(1)		1.0(1)
Leu		1.2(1)					
Phe		0 9(1)		1 0(1)		0.9(1)	
Lys	1 0(1)	1.0(1)	0.9(1)	1 0(1)		0.9(1)	1.2(1)
Arg				1.0(1)			1.1(1)
Trp					+ (1)		+ (1)
Total	4	10	5	8	8	5	6
Yield(%)	26	25	30	26	22	17	11
Charge	+1	-2	+1	+1	-2	+1	+2

Table XII Amino acid composition of the chymotryptic peptides of CB 4
Peptides were purified by peptide mapping

	Peptides							
	C 1	C 2	C 2a	C 2b	C 3	C 4	C 5	C 6
Asp					0 9(1)		1 0(1)	0 8(1)
Thr						1 0(1)		
Ser	0 9(1)	2 2(2)	1 9(2)		1 9(2)		2 1(2)	
Hse								1 0(1)
Glu		2 6(3)	1 5(2)	1 3(1)	0 8(1)			
Pro		1 9(2)	1 7(2)		3 2(3)			
Gly	1 1(1)	1 2(1)	1 0(1)		1 0(1)	2 2(2)	2 1(2)	
Ala		1 3(1)	1 1(1)		1 1(1)		0 6(1)	2 1(2)
Val		2 2(2)	1 9(2)		1 0(1)	0 9(1)		
Ile		0 9(1)		0 7(1)		0 9(1)	1 8(2)	
Leu	0 9(1)	1 2(1)	1 1(1)		1 0(1)			
Tyr		1 1(1)		0 8(1)				
Phe	1 1(1)	1 2(1)	0 9(1)		1 0(1)	1 0(1)		
His		0 9(1)		0 8(1)				
Lys		1 1(1)	1 0(1)		1 0(1)	1 0(1)	0 9(1)	
Total	4	17	13	4	13	7	9	4
Yield(%)	38	25	16	7	32	34	21	53
Charge	0	-1	-1	0	0	+1	0	0

Table XIII Amino acid composition of the thermolytic peptides of CB 4
Peptides were purified by peptide mapping

	Peptides						
	Th 1	Th 2	Th 3	Th 4	Th 5	Th 5a	Th 5b
Asp					1 0(1)	1 0(1)	
Ser	1 1(1)	0 9(1)	0 8(1)		1 7(2)	1 6(2)	
Glu			2 2(2)	1 1(1)	1 2(1)	1 0(1)	
Pro			2 3(2)		3 0(3)	2 7(3)	
Gly	1 0(1)		1 1(1)		1 2(1)	1 2(1)	
Ala			1 1(1)		1 3(1)	1 1(1)	
Val			1 9(2)		0 9(1)		0 9(1)
Ile					0 7(1)	0 6(1)	
Leu		1 1(1)		0 9(1)	1 1(1)		0 9(1)
Tyr					1(1)	1 0(1)	
Phe	0 9(1)		0 9(1)		1 0(1)		1 2(1)
His					0 8(1)	0 8(1)	
Lys			0 9(1)		1 1(1)	1 0(1)	
Total	3	2	11	2	16	13	3
Yield(%)	42	34	23	37	13	4	5
Charge	0	0	-1	-1	+1	+1	0

	Peptides						
	Th 6	Th 7	Th 7a	Th 7b	Th 8	Th 8a	Th 9
Asp		1 1(1)		1 1(1)			1 0(1)
Thr		0 9(1)	0 9(1)				
Ser		1 9(2)		1 7(2)			
Hse							1 0(1)
Gly	2 0(2)	2 7(2)		2 3(2)			
Ala					1 1(1)		2 0(2)
Val	0 9(1)						
Ile		2 3(2)	1 1(1)	1 1(1)	0 9(1)	0 9(1)	
Phe		1 1(1)		0 8(1)			
Lys	1 0(1)				1 0(1)	1 1(1)	
Total	4	9	2	7	3	2	4
Yield ( )	50	19	8	6	25	6	25
Charge	+1	-1	0	-1	+1	+1	0

Table XVI Amino acid composition of the thermolytic peptides of CB 8
Peptides were purified by peptide mapping

	Peptides								
	Th 1	Th 1a	Th 2	Th 3	Th 4	Th 5	Th 6	Th 7	Th 8
Asp	2 8(3)	0 8(1)	2 9(3)	1 0(1)			1 0(1)		
Thr	0 9(1)	0 8(1)	1 0(1)			1 1(1)		1 0(1)	1 0(1)
Ser	0 8(1)								
Hse									1 0(1)
Glu			1 9(2)						
Pro	2 0(2)						1 0(1)		
Gly	3 0(3)	0 9(1)	1 0(1)						1 0(1)
Ala	1 7(2)	1 0(1)	1 0(1)	1 9(2)		0 9(1)		0 9(1)	1 2(1)
Cys					1 0(1)[a]				
Val	1 7(2)[b]		0 9(1)				1 0(1)		
Ile			1 0(1)	0 9(1)					
Leu			1 0(1)	1 2(1)	1 0(1)				1 0(1)
Tyr					1 0(1)				
Phe							1 0(1)		
His						1 0(1)			
Lys	2 6(3)	1 0(1)					1 0(1)		
Arg	1 8(2)	1 0(1)	1 0(1)						
Total	19	6	12	5	3	3	5	2	5
Yield (%)	40	11	25	11	54	43	19	33	30
Charge	+3	+2	-2	-1	+1	+1	+1	0	0

[a]Determined as S aminoethylcysteine
[b]Value taken from the 72 h hydrolysate

Table XIV Amino acid composition of the tryptic peptides of CB 8
Peptides were purified by peptide mapping

	Peptides						
	T 31b	T 32	T 33	T 34	T 35	T 36a	T 36b
Asp		2 0(2)		0 9(1)	3 3(3)	1 2(1)	1 1(1)
Thr					1 6(2)		1 2(1)
Ser	0 9(1)						
Glu					1 8(2)		
Pro	0 8(1)	1 0(1)					1 0(1)
Gly	1 2(1)	1 2(1)		1 1(1)	1 0(1)		
Ala		0 9(1)	1 0(1)		1 1(1)	1 9(2)	1 1(1)
Cys						1 0(1)[a]	
Val		1 4(2)			1 0(1)		
Ile					1 0(1)	1 0(1)	
Leu						2 9(3)	
Tyr							0 8(1)
Phe							1 0(1)
His							1 0(1)
Lys	1 0(1)	1 0(1)		1 0(1)			1 0(1)
Arg		1 2(1)	1 0(1)		1 1(1)		
Total	4	9	2	3	12	8	8
Yield (%)	33	39	40	19	32	19	34
Charge	+1	+1	+1	+1	-2	0	+2

[a]Determined as S aminoethylcysteine

Table XVII Amino acid composition of the chymotryptic peptides of CB 9 and the thermolytic peptides of CB 9-C 1
Peptides were purified by peptide mapping

	Peptides						
	C 1	C 2	C 1-Th 1	C 1-Th 2	C 1-Th 3	C 1-Th 3a	C 1-Th 4
Asp	0 9(1)	1 2(1)	0 8(1)				
Thr	0 8(1)	1 0(1)		0 6(1)	0 6(1)	1 1(1)	
Ser	0 7(1)	1 8(2)		1 1(1)			
Gly	3 2(3)			3 3(3)	1 3(1)	1 0(1)	
Ala	2 9(2)		1 1(1)	0 7(1)	0 9(1)	0 9(1)	
Val	1 0(1)				1 2(1)		0 9(1)
Ile	1 2(1)		1 0(1)				
Leu	1 2(1)			1 2(1)			
Phe	1 0(1)				1 0(1)		1 1(1)
Trp		+ (1)					
Total	12	5	3	7	5	3	2
Yield (%)	23	16	38	35	6	20	30
Charge	-1	0	-1	0	0	0	0

Table XV Amino acid composition of the chymotryptic peptides of CB 3
Peptides were purified by peptide mapping

	Peptides				
	C 1	C 2	C 3	C 4	C 5
Asp	2 8(3)	1 0(1)			
Thr	1 9(2)			0 9(1)	0 9(1)
Hse					1 0(1)
Glu	2 0(2)				
Gly	1 0(1)				1 0(1)
Ala	1 1(1)	1 9(2)		1 0(1)	1 2(1)
Cys			0 9(1)[a]		
Val	0 8(1)				
Ile	0 8(1)	0 8(1)			
Leu	1 0(1)	1 9(2)			
Tyr			1 1(1)		
Phe				1 0(1)	
His				0 9(1)	
Arg	1 0(1)				
Total	13	6	2	4	4
Yield (%)	7	6	20	23	27
Charge	2	-1	+1	+1	0

[a]Determined as S-aminoethylcysteine

Table XVIII Amino acid composition of the tryptic and chymotryptic peptides of CB 10. Peptides were purified by peptide mapping.

	Peptides					
	T 38	T 39	T 40a	C 1	C 2	C 3
Asp	1 1(1)			0.9(1)	0.9(1)	
Thr	0 9(1)				1 0(1)	
Ser	0 9(1)				0.9(1)	
Hse			1.0(1)			1.0(1)
Glu	2.1(2)				2.1(2)	
Gly	1 0(1)				1.1(1)	
Ala	1 1(1)				1.2(1)	
Val		0 9(1)			1.0(1)	
Ile	1 0(1)				1.1(1)	
Leu	0 9(1)			1.1(1)		
Phe	1 0(1)			1 1(1)		
Lys				0 9(1)		
Arg	1 0(1)	1.0(1)			1.0(1)	1.0(1)
Trp		+ (1)			+ (1)	
Total	11	3	1	4	11	2
Yield (%)	20	22	10	21	29	13
Charge	-2	+1	0	+1	-2	+1

Table XIX. Amino acid composition of the tryptic and chymotryptic peptides of CB 12. Peptides were purified by peptide mapping.

	Peptides				
	T 45b	T 46	T 47a	C 1	C 2
Asp	2 0(2)			2.2(2)	
Thr	1.0(1)			1.1(1)	
Hse			1 0(1)		+ (1)
Glu	1 0(1)			1 0(1)	
Pro	0 9(1)			0.9(1)	
Gly			1 1(1)		1.0(1)
Val	1.0(1)			0.9(1)	
Leu	1.0(1)				0.8(1)
Tyr	1.1(1)			0.9(1)	
Lys	1.0(1)	1.0(1)	0 9(1)	0.9(1)	1.0(1)
Arg	1.1(1)				1 1(1)
Total	10	1	3	8	5
Yield (%)	40	4	36	24	27
Charge	0	+1	+1	-1	+2

Table XX. Amino acid composition of the cysteine containing tryptic peptides of [2-^{3}H] S-carboxymethylated leucine aminopeptidase
All peptides were purified by a combination of gel filtration (Fig. 17) and peptide mapping, except peptide T 14 which was already pure after the gel filtration procedure

	Peptides						
	T 13	T 14	T 30	T 31	T 36	T 41	T 43
Asp		4 5(5)	1 2(1)	3 0(3)	1 9(2)	4 0(4)	
Thr			1 1(1)		1 0(1)		1 0(1)
Ser		0 7(1)	2 1(2)	1 0(1)			0 9(1)
Glu		6 8(7)		1 1(1)		1.9(2)	
Pro		1 9(2)		3.1(3)	0.7(1)		
Gly	1 1(1)	3.1(3)	2.3(2)	2.1(2)		1.2(1)	1.1(1)
Ala	3.9(4)	5 0(5)	5 9(6)	1 1(1)	2.9(3)	1.2(1)	4.9(5)
Cys[a]	1 0(1)	0 8(1)	1 0(1)[b]	1 0(1)[b]	0 7(1)	0.7(1)[b]	0.8(1)
Val	1.0(1)	2.9(3)	1.1(1)[b]	0.8(1)[b]		1.9(2)[b]	
Met			1.0(1)[b]	0 8(1)[b]			
Ile		1 6(2)	1.6(2)[b]	1 7(2)[b]	1 1(1)	1.8(2)	
Leu		3 7(4)		3.7(4)	3 2(3)	1.1(1)	1.1(1)
Tyr		1.8(2)			0 9(1)		
Phe					1.0(1)		1 0(1)
His					1 0(1)		
Lys		1 0(1)	1 3(1)	1.0(1)	1 0(1)	1 1(1)	1 0(1)
Arg	1 1(1)						
Total	8	36	18	21	16	15	12
Yield(%)	30	52	10	17	8	4	10
Charge	0	n d	-1	-2	0	-2	0

n.d. = not determined

[a] determined as S-carboxymethylcysteine
[b] value taken from the 72 h hydrolysate

Table XXII Amino acid composition of the cysteine containing tryptic peptides of S-aminoethylated CB 1
Peptides were isolated by peptide mapping

	Peptides		
	T 13	T 14a	T 14b
Asp		2 1(2)	2 9(3)
Ser		0 9(1)	
Glu		4 2(4)	3 2(3)
Pro		2 0(2)	
Gly	0 9(1)		2 7(3)
Ala	4 1(4)		5 1(5)
Cys	1 0(1)[a]	0 7(1)[a]	
Val	1 0(1)	1 9(2)	1 1(1)
Ile		1 9(2)	
Leu		1 1(1)	2 5(3)
Tyr			1 7(2)
Lys			1 1(1)
Arg	1 0(1)		
Total	8	15	21
Yield (%)	9	8	6
Charge	+2	> 2	> -3

[a]determined as S-aminoethylcysteine

Table XXIII Amino acid composition of HA 2
Data are given as numbers of residues per polypeptide chain as determined by amino acid analysis and sequence results Values for threonine and serine were extrapolated to zero time hydrolysis, and values for valine and isoleucine were taken from the 72 h hydrolysate

Amino acid	Composition	Sequence
Aspartic acid	19 0	18
Threonine	12 2	13
Serine	7 1	7
Glutamic acid	11 2	10
Proline	4 9	5
Glycine	11 7	12
Alanine	19 7	20
Cysteine	2 7[a]	3
Valine	8 3	9
Methionine	3 5	5
Isoleucine	8 0	9
Leucine	12 2	11
Tyrosine	3 3	4
Phenylalanine	7 4	7
Histidine	4 2	4
Lysine	8 5	8
Arginine	3 4	8
Tryptophan	n d	3
Total	152 3	156

n d = not determined

[a]determined as S-aminoethylcysteine

Table XX' Amino acid composition of peptides obtained by chymotryptic (C), thermolytic (Th) or staphylococcal protease (SP) digestion of some cysteine containing tryptic peptides of leuc ne aminopeptidase
Peptides were purified by peptide mapping

	Peptides																	
	T 14-C 1	T 14-C 1	T 14-C 2	T 14-C 2-3	T 14-C 3	T 14- SP 1	T 14-SP 1	T 14-SP 1-2	T 14-SP 3	T 14-SP 4	T 14-SP5
Asp	2 8(3)		2 1(2)	2 0(2)	1 2(1)	1 1(1)		2 2(2)		1 6(2)	
Ser	0 8(1)					1 0(1)					
Glu	5 6(6)		1 1(1)	0 9(1)	2 8(3)	4 0(4)		2 2(2)	1 3(1)		
Pro	2 0(2)					0 8(1)		1 1(1)			
Gly	2 0(2)	0 9(1)	1 0(1)					1 1(1)	1 8(2)		
Ala	4 7(5)							4 2(4)	1 4(1)		
Cys	0 8(1)[a]							0 8(*)[a]			
Val	2 6(3)					1 2(1)		1 0(1)	0 8(1)		
Ile	1 9(2)				1 0(1)	1 7(2)					
Leu	1 9(2)	1 0(1)	1 8(2)	0 9(1)	1 0(1)	1 1(1)			1 9(2)	1 2(1)	
Tyr		1 0(1)	1 9(2)	1 0(1)					0 9(1)	1 0(1)	
Lys			1 1(1)	1 0(1)						1 2(1)	
Total	27	3	9	6	6	11		12	8	5	
Yield (%)	n d	n d	n d	n d	4	12		27	12	27	
Charge	-3	0	-2	-2	-2	3		-3	-1	-1	

	Peptides									
	T 31-Th 1	T 31- Th 1	T 31 Th 2	T 31-Th 3	T 31-Th 4	T 31-Th 4a	T 41-Th 1	T 41-Th 1	T 41-Th 2	T 41-Th 3
Asp	1 1(1)	1 1(1)		1 3(1)	1 3(1)	1 0(1)	3 2(3)			
Ser				0 9(1)	0 9(1)					
Glu				1 2(1)	1 1(1)	1 8(2)				
Pro	0 9(1)		1 0(1)	2 0(2)	1 1(1)					
Gly			1 3(1)	1 1(1)	1 1(1)				1 0(1)	
Ala			1 1(1)	1 2(1)				1 0(1)		
Cys				1 0(1)[a]	1 0(1)[a]	0 8(1)[b]				
Val			0 8(1)			0 6(1)	1 1(1)			
Met				0 7(1)	0 7(1)					
Ile		0 9(1)	0 6(1)			0 5(1)			0 8(1)	
Leu	1 5(2)		1 0(1)	1 7(2)	0 8(1)			0 7(1)		
Lys				1 0(1)	1 0(1)				1 2(1)	
Total	4	2	6	12	9	6	6	3		
Yield (%)	80	90	70	24	34	6	18	27		
Charge	-1	0	0	-1	-1	0	-1	+1		

n d not determined

[a]determined as S carboxymethylcysteine
[b]determined as S-aminoethylcysteine

Table XXIV Automatic Edman degradation of S-aminoethylated HA 2
Approximately 0 45 μmol of sample was subjected to automatic Edman degradation in a Beckman sequencer Residues were identified as phenylthiohydantoin by thin layer chromatography and quantified by gas chromatography The repetitive yield was 94

Cycle	Residue	Yield (nmol)
1	Gly	300
2	Lys	290
3	Thr	270
4	Ile	265
5	Gln	
6	Val	245
7	Asp	
8	Asn	
9	Thr	210
10	Asp	
11	Ala	200
12	Glu	
13	Gly	180
14	Arg	
15	Leu	170
16	Ile	160
17	Leu	155
18	Ala	150
19	Asp	
20	Ala	140
21	Leu	130
22		
23	Tyr	
24	Ala	115
25	His	
26	Thr	100
27	Phe	100
28	Asn	

Table XXV Amino acid composition of the tryptic peptides of CB 8b
All peptides were purified by peptide mapping Peptides T 36a and T 37a were further separared by re-electrophoresis at pH 3 5

	Peptides				
	T 34b	T 35	T 36a	T 36b	T 37a
Asp		3 0(3)	1 0(1)	1 0(1)	1 1(1)
Thr		1 9(2)		1 0(1)	1 7(2)
Hse					1 0(1)
Glu		2 1(2)			
Pro				1 3(1)	
Gly	1 0(1)	1 0(1)			1 2(1)
Ala		1 1(1)	2 1(2)	1 0(1)	3 2(3)
Cys			1 0(1)[a]		
Val		0 9(1)			0 9(1)[b]
Ile		0 9(1)	1 0(1)		2 0(2)[b]
Leu			2 9(3)		1 2(1)
Tyr				0 9(1)	
Phe				1 0(1)	
His				1 0(1)	
Lys	1 0(1)			1 0(1)	
Arg		1 1(1)			
Total	2	12	8	8	12
Yield ( )	28	25	14	25	7
Charge	+1	-2	0	+2	0

[a] determined as S-aminoethylcysteine
[b] value taken from the 72 h hydrolysate

Table XXVI Amino acid compostion of the thermolytic peptides of CB 8b
Peptides were purified by peptide mapping

	Peptides										
	Th 1	Th 2	Th 3	Th 4	Th 5	Th 6a	Th 6	Th 7a	Th 7	Th 8	Th 9
Asp		2 9(3)	1 0(1)			1 0(1)	1 1(1)	1 1(1)	1 1(1)		
Thr	1 1(1)	1 2(1)			1 1(1)					1 1(1)	1 0(1)
Hse											1 0(1)
Glu		2 1(2)									
Pro						1 0(1)	0 8(1)				
Gly	0 9(1)	0 9(1)									1 0(1)
Ala		1 0(1)	1 9(2)		0 9(1)			1 0(1)	1 0(1)	0 9(1)	1 0(1)
Cys				1 0(1)[a]							
Val		0 9(1)					1 0(1)		0 9(1)[b]		
Ile		1 0(1)	1 0(1)					1 9(2)[b]	2 0(2)[b]		
Leu		1 0(1)	1 1(1)	1 0(1)							1 0(1)
Tyr				1 0(1)							
Phe						1 0(1)	1 0(1)				
His					1 0(1)						
Lys	1 0(1)					1 0(1)	1 1(1)				
Arg		1 0(1)									
Total	3	12	5	3	3	4	5	4	5	2	5
Yield(%)	33	45	49	54	43	14	13	37	20	83	67
Charge	+1	-2	-1	+1	+1	+1	+1	0	0	0	0

[a] determined as S aminoethylcysteine
[b] value taken from the 72 h hydrolysate

Table XXIX Amino acid composition of the thermolytic peptides of CB 11
Peptides were isolated by gel filtration followed by peptide mapping

	Peptides					
	Th 1	Th 2	Th 3	Th 4	Th 5	Th 6
Asp			1 0(1)	2 7(3)		
Thr		0 9(1)				1 0(1)
Ser					0 8(1)	
Glu	1 0(1)	1 1(1)	1 0(1)			
Gly					1 1(1)	1 1(1)
Ala				1 0(1)		2 8(3)
Cys			0 3(1)[a]			0 8(1)[a]
Val			0 6(1)	1 1(1)		
Ile			0 6(1)		0 9(1)	
Leu	0 9(1)			1 0(1)		
Tyr	1 0(1)				1 1(1)	
Phe	1 0(1)					
His	1 1(1)					
Lys					1 0(1)	
Arg		1 0(1)			1 0(1)	
Total	5	3	5	6	6	6
Yield ( )	28	33	34	23	22	25
Charge	0	+1	-2	-1	+2	-1

	Peptides					
	Th 7	Th 8	Th 9	Th 10	Th 11	Th 12
Asp				1 0(1)	1 0(1)	
Thr			1 0(1)			
Hse						1 0(1)
Glu		1 0(1)				
Pro			1 0(1)			
Gly					1 0(1)	
Ala	1 9(2)			1 0(1)	1 0(1)	
Val			1 0(1)			1 0(1)
Ile					1 0(1)	
Leu		0 8(1)		1 0(1)	0 9(1)	
Phe	1 0(1)	1 1(1)				
His			1 1(1)	1 0(1)		
Lys		1 0(1)	1 0(1)			
Trp			+ (1)			
Total	3	4	6	4	5	2
Yield (%)	36	16	21	5	26	0
Charge	0	0	+2	0	-1	0

[a]determined as S-carboxymethylcysteine

Table XXVII Amino acid composition of the tryptic peptides of CB 11
Peptides were isolated by peptide mapping

	Peptides							
	T 40b	T 41	T 41b	T 42	T 43a	T 43b	T 44	T 45a
Asp		4 6(4)	2 9(3)					1 0(1)
Thr	0 8(1)					0 9(1)	1 0(1)	
Ser					0 7(1)			
Hse								1 0(1)
Glu	1 2(1)	1 7(2)	1 4(1)				0 9( )	
Pro	0 8(1)						0 9(*)	
Gly		1 0(1)	0 9(1)		1 0(1)			1 0(1,
Ala		1 1(1)	1 0(1)		2 2(2)	2 8(3)		2 1(2)
Cys		1 0(1)[a]			1 1(1)[a]			
Val		1 6(2)	1 0(1)				1 1(1)	1 1(1)
Ile		2 0(2)	1 1(1)					1 0(1)
Leu	1 0(1)	1 0(1)	0 8(1)			1 0(1)		1 1(1)
Tyr	1 1(1)			1 1(1)				
Phe	1 1(1)					1 3(1)	1 1(1)	
His	1 2(1)						1 1(1)	1 0(1)
Lys		1 1(1)	1 0(1)			1 0(1)	0 9(1)	
Arg	0 9(1)			0 9(1)				
Trp								+ (1)
Total	8	15	10	2	5	7	7	10
Yield( )	16	20	5	75	38	42	56	15
Charge	+1	0	0	+1	+1	+1	+	+1

[a]determined as S-aminoethylcysteine

Table XXVIII Amino acid composition of the chymotryptic peptides of CB 11
Peptides were isolated by peptide mapping

	Peptides							
	C -2	C 1	C 2	C 3	C 4	C 5	C 5a	C 6
Asp	3 8(4)	3 3(3)	1 0(1)					1 0(1)
Thr	0 9(1)	1 1(1)		0 9(1)		0 8(1)		
Ser				0 9(1)				
Hse								1 0(1)
Glu	2 0(2)	2 1(2)			0 9(1)			
Pro						1 0(1)		
Gly	1 0(1)		1 0(1)	1 0(1)				1 0(1)
Ala	1 0(1)	1 2(1)		4 3(5)		1 0(1)	1 0(1)	1 1(1)
Cys	0 8(1)[a]	0 8( )[a]		0 9(1)[a]				
Val	1 6(2)	1 6(2)				0 9(1)		0 9(1)
Ile	1 6(2)	0 7(1)	1 0( )					0 9(1)
Leu	1 0(1)	1 0(1)			0 8(1)	1 0(1)	1 0(1)	
Tyr	0 8(1)		0 9(1)					
Phe				1 0(1)	1 0(1)			
His						1 9(2)	1 0(1)	
Lys	1 0(1)		1 0(1)		1 0(1)	1 0(1)		
Arg	0 9(1)	1 0(1)		1 0(1)				
Trp						+ (1)		
Total	18	13	5	11	4	9	3	6
Yield(%)	12	10	10	16	27	14	10	16
Charge	+1	0	+1	+2	0	+2	+1	-1

[a]determined as S-aminoethylcysteine

Table XXX Amino acid composition of the staphylococcal peptides of CB 11
Peptides were purified by peptide mapping

	Peptides					
	SP 1	SP 2	SP 3	SP 4	SP 5	SP 6
Asp	1 0(1)	0 9(1)	2 2(2)		1 0(1)	
Thr	0 8(1)		0 9(1)		0 9(1)	
Ser			0 3(1)			
Hse						1 0(1)
Glu	0 9(1)	1 0(1)		1 0(1)		
Pro					1 2(1)	
Gly			2 3(2)			1 0(1)
Ala		1 1(1)	3 0(3)	1 9(2)	1 0(1)	1 1(1)
Cys		0 8(1)[a]	0 9(1)[a]			
Val	0 5(1)		1 2(1)		1 0(1)	0 9(1)
Ile	0 5(1)		1 0(1)			0 9(1)
Leu		1 0(1)		1 0(1)	1 0(1)	
Tyr	1 2(1)		1 1(1)			
Phe				1 0(1)	0 8(1)	
His	0 9(1)				2 1(2)	
Lys			1 0(1)	1 0(1)	1 1(1)	
Arg	1 0(1)		1 0(1)			
Trp					+ (1)	
Total	8	5	15	6	11	5
Yield (%)	20	18	10	12	22	21
Charge	+1	-2	+1	0	+2	0

[a]determined as S-carboxymethylcysteine

Table XXXI Amino acid composition of the tryptic peptides of staphylococcal protease peptide $SP^{232-304}$

Peptides were purified by peptide mapping

	Peptides						
	T 26b	T 27	T 28	T 29	T 30a	T 30b	T $31^{290-304}$
Asp		1 1(1)	1 3(1)	2 3(2)	1 3(1)		2 3(2)
Thr			1 1(1)		1 1(1)		
Ser		1 7(2)	2 2(2)			1 7(2)	
Glu		1 1(1)					1 1(1)
Pro		3 0(3)					2 3(2)
Gly		2 0(2)	2 9(3)		1 9(2)		1 2(1)
Ala		1 4(1)		2 6(3)	3 0(3)	3 1(3)	1 4(1)
Cys					1 0(1)[a]		1 0(1)[a]
Val		1 8(2)				0 9(1)	0 7(1)
Met				1 8(2)	0 9(1)		
Ile	0 8(1)		2 6(3)		1 0(1)	0 7(1)	1 6(2)
Leu		1 1(1)		1 1(1)			4 2(4)
Tyr	1 1(1)						
Phe		0 9(1)	1 1(1)				
His	0 9(1)						
Lys	1 1(1)	0 8(1)	1 1(1)			1 0(1)	
Arg				1 1(1)			
Total	4	15	12	9	10	8	15
Yield (%)	35	43	39	34	34	27	17
Charge	+2	0	0	0	0	+1	-1

[a]determined as S-aminoethylcysteine

Table XXXII Amino acid composition of the chymotryptic peptides of staphylococcal protease peptide $SP^{232-304}$

Peptides were isolated by gel filtration followed by peptide mapping

	Peptides						
	C 1	C 2	C 3	C 4	C 4a	C 4b	C 4b-5
Asp		1 2(1)		1 6(2)	1 1(1)	1 1(1)	2 1(2)
Thr			0 9(1)				
Ser		1 8(2)		1 8(2)	1 8(2)		
Glu		1 2(1)					
Pro		2 7(3)					
Gly		1 2(1)	2 2(2)	3 4(2)	2 2(2)		
Ala		1 2(1)		2 2(3)		3 1(3)	2 9(3)
Val		0 8(1)	1 0(1)				
Met				0 6(1)		0 7(1)	1 3(2)
Ile	1 0(1)		0 9(1)	2 3(2)	1 9(2)		
Leu		1 1(1)					0 9(1)
Tyr	1 1(1)						
Phe		0 8(1)	0 9(1)				
His	0 9(1)						
Lys		1 0(1)	1 1(1)	1 0(1)	0 9(1)		
Total	3	13	7	13	8	5	8
Yield (%)	10	23	26	9	12	7	3
Charge	+1	0	+1	0	0	0	-1

	Peptides						
	C 5	C 6	C 7	C 7a	C 7b	C 8	C 9
Asp	1 1(1)	1 1(1)				1 9(2)	
Thr		0 9(1)					
Ser			1 6(2)				
Glu							1 1(1)
Pro						2 2(2)	
Gly		2 2(2)				1 2(1)	
Ala		3 2(3)	3 2(3)	1 2(1)		1 3(1)	
Cys		1 0(1)[a]					0 9(1)[a]
Val			0 6(1)			0 7(1)	
Met	0 9(1)	0 9(1)					
Ile		0 9(1)	0 6(1)			1 5(2)	
Leu	1 0(1)		0 9(1)	0 9(1)	1 0(1)	3 1(3)	
Lys			1 0(1)	0 9(1)	1 0(1)		
Arg		0 7(1)					
Total	3	11	9	3	2	12	2
Yield (%)	6	4	4	8	3	14	19
Charge	-1	+1	+1	+1	+1	1	0

[a]determined as S-aminoethylcysteine

Table XXXV. Amino acid composition of the chymotryptic peptides of fraction 1 (Fig 20) of a tryptic digest of citraconylated HA 2.
Peptides were isolated by peptide mapping.

	Peptides					
	Tc 1-C 1	Tc 1-C 2	Tc 1-C 3	Tc 1-C 4	Tc 1-C 5	Tc 1-C 6
Asp			0.8(1)	0.9(1)	1 2(1)	0 9(1)
Thr		1.0(1)		0 7(1)	1 0(1)	
Ser					1 8(2)	
Pro			1.1(1)			
Ala		1 2(1)		1.8(2)		
Cys	0.9(1)[a]					
Val			1.0(1)			
Ile				1 0(2)		
Leu				0.8(1)		1 0(1)
Tyr	1.1(1)					
Phe		1.0(1)				1.1(1)
His		0 9(1)				
Lys			1 0(1)			1.0(1)
Trp					+ (1)	
Total	2	4	4	7	5	4
Yield (%)	27	10	7	6	10	10
Charge	+1	+1	+1	0	0	+1

	Peptides					
	Tc 1-C 7	Tc 4-C 1	Tc 4-C 2	Tc 4-C 3	Tc 4-C 4	Tc 4- C 5
Asp	0.9(1)				1 8(2)	
Thr	0.7(1)	0 9(1)		0.9(1)	0.8(1)	
Ser	0.9(1)	0.8(1)				
Glu	2.1(2)		1 0(1)		1.1(1)	
Pro				1.5(1)	1 3(1)	
Gly	1.4(1)	1.0(1)				
Ala	1.1(1)	5.5(5)				
Cys		0.7(1)[a]				
Val				0 9(1)	1.0(1)	
Ile	0.8(1)					
Leu			1 0(1)			1 0(1)
Tyr					1.0(1)	
Phe		1.0(1)	1.0(1)			
His				0.9(1)		
Lys			1.0(1)	0.9(1)	1 0(1)	
Arg	1.0(1)					1.0(1)
Trp				+ (1)		
Total	9	10	4	6	8	2
Yield (%)	18	10	21	10	12	27
Charge	-2	+1	0	+2	-1	+1

[a] determined as S-aminoethylcysteine

Table XXXIII. Amino acid composition of staphylococcal protease peptide $Sp^{232-304}$. Data are given as number of residues per polypeptide chain as determined by amino acid analysis and sequence results. Values for threonine and serine were extrapolated to zero time hydrolysis, and values for valine and isoleucine were taken from the 72 h hydrolysate.

Amino acid	Composition	Sequence
Aspartic acid	9.1	7
Threonine	2.0	2
Serine	4 7	6
Glutamic acid	4.2	2
Proline	5.2	5
Glycine	7.2	8
Alanine	10 7	11
Cysteine	1 5[a]	2
Valine	4.0	4
Methionine	2.2	3
Isoleucine	6 0	8
Leucine	5.9	6
Tyrosine	1 1	1
Phenylalanine	2.3	2
Histidine	1 0	1
Lysine	4 1	4
Arginine	1 5	1
Total	73 5	73

[a] determined as S-aminoethylcysteine

Table XXXIV. Amino acid composition of some methionine containing peptides of HA 2.
Peptides were purified by peptide mapping. The neutral peptide T 45 was subjected to re-electrophoresis at pH 3.5 for further purification

	Peptides	
	T 40	T 45
Asp		2.6 (3)
Thr	1.0 (1)	0 8 (1)
Glu	1 1 (1)	1 0 (1)
Pro	1.0 (1)	0.9 (1)
Gly		1.0 (1)
Ala		1.8 (2)
Val		1.7 (2)
Met	0.8 (1)	0.7 (1)
Ile		0.8 (1)
Leu	1 0 (1)	1.8 (2)
Tyr	0 8 (1)	0 7 (1)
Phe	1.0 (1)	
His	0.9 (1)	1.0 (1)
Lys		0.9 (1)
Arg	1.0 (1)	1.1 (1)
Trp		+ (1)
Total	9	20
Yield (%)	30	9
Charge	+1	0

Table XXXV: Amino acid composition of the thermolytic peptides of fraction I (Fig 20) of a tryptic digest of citraconylated HA 2
Peptides were purified by peptide mapping

	Peptides						
	Tc 1-Th 1	Tc 1-Th 2	Tc 1-Th 3	Tc 1-Th 4	Tc 4-Th 1	Tc 4-Th 2	Tc 4-Th 3
Asp	0.9(1)		1.1(1)				
Thr		1.2(1)	1.0(1)		1.0(1)		
Ser					0.9(1)		
Glu							1.1(1)
Pro	1.2(1)						
Gly			1.2(1)		1.1(1)		
Ala		0.8(1)	1.1(1)	1.0(1)	2.2(2)	2.5(3)	
Cys					0.9(1)[a]		
Met			0.7(1)				
Ile				1.0(1)			
Leu			0.8(1)				0.9(1)
Phe	0.9(1)					1.1(1)	1.0(1)
Lys	1.0(1)						1.0(1)
Total	4	2	6	2	6	4	4
Yield ( )	12	14	19	12	13	13	5
Charge	+1	0	-1	0	+1	0	0

	Peptides					
	Tc 4-Th 4	Tc 4-Th 5	Tc 4-Th 6	Tc 4-Th 7	Tc 4-Th 8	Tc 4-Th 9
Asp			1.1(1)		2.1(2)	
Thr	0.8(1)				1.0(1)	
Glu					1.1(1)	
Pro	1.0(1)				1.9(1)	
Gly				1.1(1)		
Ala		1.0(1)		1.1(1)		
Val	1.3(1)				1.9(2)	
Met					1.0(1)	
Ile				0.9(1)		
Leu			0.9(1)			0.8(1)
Tyr					0.9(1)	
His	0.8(1)	1.0(1)				
Lys	1.0(1)				1.1(1)	
Arg						1.2(1)
Trp	+ (1)					
Total	6	2	2	3	10	2
Yield (%)	5	27	25	17	14	42
Charge	0	+1	-1	0	-1	+1

[a]determined as S-aminoethylcysteine

Table XXXVII Amino acid composition of the tryptic peptides of citraconylated HA 2
Peptides were purified by gel filtration (Fig. 30) followed by peptide mapping of fraction III up to VI.

	Peptides			
	Tc 2	Tc 3	Tc 5	Tc 6
Asp				1.0 (1)
Thr		1.1 (1)	1.1 (1)	
Ser				1.6 (2)
Glu		1.3 (1)		1.0 (1)
Pro		0.9 (1)	1.0 (1)	
Gly			2.2 (2)	
Ala			1.1 (1)	1.0 (1)
Val	0.7 (1)			
Met		0.9 (1)	1.0 (1)	
Leu		1.0 (1)		
Tyr		1.0 (1)		
Phe		0.9 (1)		0.7 (1)
His		1.1 (1)		
Lys			0.9 (1)	
Arg	1.2 (1)	0.9 (1)	1.8 (2)	
Trp	+ (1)			
Total	3	9	9	6
Yield ( )	15	16	14	8
Charge	+1	+1	+3	-1
Fraction Fig 30	VI	IV, V, VI	IV, V	IV

Table XXXIX Amino acid composition of some thermolytic peptides of HA 2
Peptides were isolated by diagonal electrophoresis at pH 6.5 (35) (E) or by peptide mapping of a digest of performic acid oxidized HA 2 (C)

	Peptides			
	Th 1	Th 2	Th 3	Th 4
Asp	1.0(1)	1.0(1)		
Thr	1.0(1)			1.1(1)[a]
Hse	1.0(1)[a]			1.0(1)[a]
Pro			0.9(1)	
Gly	1.2(1)			
Ala	1.2(1)			
Val			0.9(1)	0.9(1)
Met		1.0(1)[b]	1.0(1)[b]	
Leu	0.7(1)	1.0(1)		
Lys		1.0(1)		
Arg			1.1(1)	
Trp		+ (1)	+ (1)	
Total	6	5	5	3
Yield ( )	21	9	14	6
Charge	-1	+1	+1	0
Purif	E	C	C	E

[a]Destruction product of Methionine
[b]Determined as Methioninesulfone

Table XL Amino acid composition of some staphylococcal peptides of HA 2
Peptides were purified by peptide mapping

	Peptides				
	SP 1	SP 2	SP 3	SP 4	SP 5
Asp	3.0(3)		1.2(1)	1.3(1)	1.0(1)
Thr	1.9(2)		1.0(1)	0.8(1)	1.2(1)
Ser		0.6(1)			
Glu	2.1(2)	1.1(1)	1.1(1)	1.1(1)	
Pro			0.9(1)		1.0(1)
Gly	1.0(1)		1.2(1)		
Ala	1.1(1)	1.3(1)			0.9(1)
Val	1.0(1)		1.0(1)	1.0(1)	1.3(1)
Met			0.8(1)		
Ile	1.0(1)	1.1(1)		0.8(1)	
Leu			1.0(1)		0.8(1)
Tyr				0.9(1)	
Phe			1.0(1)		0.7(1)
His				0.9(1)	1.4(2)
Lys	0.9(1)				0.8(1)
Arg			1.8(2)	1.2(1)	
Trp			+ (1)		+ (1)
Total	12	4	12	8	11
Yield ( )	11	32	7	4	4
Charge	-2	-1	0	+1	+1

Table XXXVIII Amino acid composition of the chymotryptic peptides of HA 2
Peptides were purified by gel filtration (A) (Fig. 31) followed by ion-exchange chromatography (B) or peptide mapping (C)

	Peptides							
	C 1	C 1a	C 2	C 3	C 4	C 5	C 6	C 7	C 8a
Asp	3.8(4)	1.0(1)		1.1(1)	1.0(1)	0.8(1)	1.2(1)		1.0(1)
Thr	2.1(2)		1.0(1)	1.9(2)	0.7(1)		1.1(1)		0.7(1)
Ser				1.0(1)	1.8(2)		0.9(1)		
Glu	2.2(2)						2.2(2)	1.0(1)	2.0(2)
Pro								1.0(1)	
Gly	1.0(2)			3.9(4)			1.0(1)		
Ala	3.0(3)	2.0(2)	1.1(1)	2.8(3)			1.0(1)		
Cys	0.9(1)[a]	0.8(1)[a]							0.9(1)[a]
Val	1.3(1)			1.0(1)			1.0(1)		0.6(1)
Met				0.9(1)				0.9(1)	
Ile	1.8(2)	0.7(1)		1.0(1)			1.0(1)		0.6(1)
Leu	2.7(3)	2.0(2)		1.0(1)		0.9(1)		1.1(1)	1.0(1)
Tyr	0.9(1)	0.9(1)						0.8(1)	
Phe			1.1(1)	0.9(1)		1.2(1)		1.1(1)	
His			1.0(1)					1.0(1)	
Lys	0.9(1)					1.0(1)			
Arg	1.0(1)						1.0(1)	1.0(1)	1.0(1)
Trp					+ (1)		+ (1)		
Total	23	8	4	16	5	4	11	8	9
Yield ( )	21	10	10	25	8	18	19	13	11
Charge	neg	2	+1	neg	0	+1	neg	+1	-1
Purif	AB	AC	AC	AB	AC	AC	AB	AC	AC

	Peptides								
	C 8	C 9	C 10	C 11	C 11a	C 12	C 13	C 14	C 15
Asp	3.9(4)					1.0(1)	2.1(2)		1.2(1)
Thr	0.8(1)	1.0(1)		1.0(1)			1.0(1)		
Ser		1.0(1)							1.8(2)
Glu	1.9(2)		0.9(1)				1.4(1)		0.8(1)
Pro				0.8(1)			1.1( )		
Gly	1.0(1)	0.9(.)				0.9(1)		1.0(1)	
Ala	0.9(1)	4.7(5)		1.0(1)	1.0(1)	1.1(1)			0.8(1)
Cys	0.8(1)[a]	0.9(.)[a]							
Val	1.7(2)			0.9(1)		0.9(1)	1.9(1)		
Met						0.7(1)		1.0(1)	
Ile	1.6(2,					1.0(1)			
Leu	1.0(1,		0.9(1)	1.0(1)	1.0(1)			1.0(1)	
Tyr	0.9(1)						1.0(1)		
Phe		1.1(1)	1.0(1)						
His				1.9/2)	1.0(1)				
Lys	1.0(1)		1.0(1)	1.1(1)			1.0(1)	1.0(1)	
Arg	0.9(1)	0.9(1)						1.0(1)	
Trp				+ (1)					
Total	18	11	4	9	3	6	8	5	5
Yield ( )	17	25	28	10	12	10	23	6	12
Charge	-1	0	0	+2	+1	1	neg	+2	-1
Purif	AC	AC	AC	AC	AC	AC	AB	AC	AC

[a]determined as S carboxymethylcysteine

Table XLI Amino acid composition of the peptides of a tryptic (T) and a staphylococcal protease (SP) digestion of fragment $Tc^{170-205}$

Peptides were purified by peptide mapping

	Peptides								
	T 22	T 23	T 24	SP 1	SP 2	SP 3	SP 4	SP 5	SP 6
Asp	1 4(1)		1 2(1)	1 3(1)			0 6(1)	1 3(1)	
Thr			0 8(1)	1 2( )	2 1(2)			0 8(1)	
Ser		1 6(2)						1 4(2)	
Glu	3 5(3)			2 5(2)	1 3(1)	2 5(2)			
Pro			1 3(1)	1 0(1)	1 0(1)				1 0(1)
Ala	1 1(1)	1 3(1)		1 0(1)	1 3(1)			1 1(1)	
Val	0 6(1)		1 1(1)			0 9(1)			0 7(1)
Met				1 1(1)	0 7(1)				
Ile	0 6(1)	1 3(1)	1 0(1)			0 6(1)		0 8(1)	1 0(1)
Leu	1 2(1)			0 9(1)			1 2(1)		
Phe	0 6(1)		1 1(1)		1 1(1)				1 1(1)
Lys	1 0(1)	1 0(1)	1 1(1)		1 0(1)		1 2(1)	0 9(1)	1 2(1)
Arg			0 7(1)						1 0(1)
Total	10	5	8	8	8	4	3	7	6
Yield ( )	25	7	8	6	5	9	5	5	8
Charge	-2	+1	+1	-2	0	-2	+1	0	+2

Table XLII Amino acid composition of peptides establishing the overlap of cyanogen bromide fragments

Peptide $T^{172-182}$ was purified by peptide mapping and peptide T 25 by gel filtration followed by peptide mapping

	Peptides	
	$T^{172-182}$	T 25
Asp	1 0 (1)	
Thr	2 6 (3)	
Ser		2 6 (3)
Hse	1 0 (1)	
Glu	2 0 (2)	4 2 (4)
Pro	2 1 (2)	
Gly		1 4 (1)
Ala	1 4 (1)	1 3 (1)
Val		1 0 (1)
Met		0 7 (1)
Ile		1 1 (1)
Leu		1 2 (1)
Phe		1 1 (1)
Lys	1 2 (1)	1 4 (1)
Trp		+ (1)
Total	11	16
Yield (%)	9	7
Charge	-1	-2

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Chapter III

SULFHYDRYL CONTENT OF BOVINE EYE LENS LEUCINE AMINOPEPTIDASE

Determination of the reactivity of the sulfhydryl groups of the zinc metalloenzyme, of the enzyme activated by Mg^{2+}, Mn^{2+}, and Co^{2+}, and of the metal-free apoenzyme

SUMMARY

The reactivity of the sulfhydryl groups of leucine aminopeptidase from bovine eye lens has been studied by carboxymethylation with iodoacetate of the native enzyme (Zn^{2+}-Zn^{2+}), of the enzyme activated with magnesium (Zn^{2+}-Mg^{2+}) or manganese (Zn^{2+}-Mn^{2+}), the enzyme incubated with cobalt (Co^{2+}-Co^{2+}), and the metal-free apoenzyme. The reactivity of the sulfhydryl groups was also determined in the presence of denaturing agents with or without reducing agents. All seven half-cystines per leucine aminopeptidase subunit are in the sulfhydryl form. In the native and the Zn^{2+}-Mn^{2+} enzyme, only one of the cysteines (residue 344) reacts readily with iodoacetate. In the Zn^{2+}-Mg^{2+} enzyme, two cysteines react (residues 344 and 412) and in the Co^{2+}-incubated enzyme only one (residue 412). The metal-free apoenzyme has at least three reactive cysteines (residues 344, 412, and 429). The data suggest that sulfhydryl groups are involved in the binding of metal ions.

INTRODUCTION

Leucine aminopeptidase from bovine lens (EC 3.4.11.1) has been characterized as a metalloenzyme containing 2 zinc ions/subunit of M_r = 52,000 (or 12 zinc ions in the native hexameric enzyme) as essential metal ions[1]. Intersubunit disulfide bridges are absent[2]. Incubation of the native enzyme, containing only Zn^{2+} ions, with various concentrations of Mg^{2+} or Mn^{2+} showed that there is a competition between the ions for one binding site, the activation site, and that a maximum of 1 mol of Mg^{2+} or Mn^{2+} is bound per 52,000 g of leucine aminopeptidase[1,3]. The other site, normally occupied by zinc, is called the structural site[1,4]. Prolonged incubation of the Zn^{2+}-Zn^{2+} enzyme with Co^{2+} yields an active enzyme in which 2 g atoms of Co^{2+}/subnit have replaced the Zn^{2+} ions[4]. Removal of zinc by dialysis against *o*-phenanthroline yields

a zinc-free product with no enzymatic activity, which upon readdition of Zn^{2+} regains full activity[1,5].

Early reports of the amino acid composition of the subunits of leucine aminopeptidase indicated that the enzyme contains 8 half-cystines/52,000 dalton subunit. These results were based on amino acid analysis of performic acid-oxidized enzyme[1] and determination of the number of titratable sulfhydryl groups using Ellman's reaction on the enzyme after denaturation in guanidine hydrochloride and reduction with $NaBH_4$[6]. Chemical modification reactions with 5,5'-dithiobis-(2-nitro-benzoic acid) (Ellman's reagent), *p*-chloromercuribenzoate and iodoacetamide on the native enzyme, the enzyme incubated with metal ions, and the enzyme treated with guanidine hydrochloride could be interpreted as follows:

1. each subunit contains one intrasubunit disulfide bond and six sulfhydryl groups[1,6]
2. one sulfhydryl group is located at the protein surface and has no catalytic function[1,7,8]. Blocking of this free sulfhydryl group reduces the rate of activation by temperature elevation. It is probably that this free sulfhydryl group is involved in an essential intermediate step of the activation[9]
3. supplementary sulfhydryl groups become free during activation by Mg^{2+} ions[10]
4. one sulfhydryl group is involved in Zn^{2+} binding[6]
5. two masked sulfhydryl groups differing in reactivity and accessibility are buried even in the apoenzyme[6].

Recently, we have determined the primary structure of bovine eye lens leucine aminopeptidase, as described in the accompanying report[11]. Our results revealed the presence of only 7 half-cystine residues/subunit. This number was obtained from amino acid analysis and from the sum of the alkylated cysteines determined during sequence analysis (see Fig. 1). We now describe the determination of the reactivity of the individual sulfhydryl groups toward iodo-[2-^{3}H]acetate and the enzymatic activity of the products of Zn^{2+}-ion readdition on the apoenzyme and the carboxymethylated apoenzyme. The chemical properties of the individual cysteine residues containing the ^{3}H-labeled carboxymethyl group are discussed in respect to their possible functions in metal ion binding.

Peptide	Sequence
T 13	91 Ala-Ala-Val-Ala-Ala-Gly-Cys-Arg
T 14	100 110 Gln-Ile-Gln-Asp-Leu-Glu-Ile-Pro-Ser-Val-Glu-Val-Asp-Pro-Cys-Gly-Asp- 120 130 Ala-Gln-Ala-Ala-Ala-Glu-Gly-Ala-Val-Leu-Gly-Leu-Tyr-Glu-Tyr-Asp-Asp- Leu-Lys
T 30	280 Ala-Asp-Met-Gly-Gly-Ala-Ala-Thr-Ile-Cys-Ser-Ala-Ile-Val-Ser-Ala-Ala- Lys
T 31	290 300 Leu-Asp-Leu-Pro-Ile-Asn-Ile-Val-Gly-Leu-Ala-Pro-Leu-Cys-Glu-Asn-Met- 310 Pro-Ser-Gly-Lys
T 36	340 350 Leu-Ile-Leu-Ala-Asp-Ala-Leu-Cys-Tyr-Ala-His-Thr-Phe-Asn-Pro-Lys
T 41	410 420 Gln-Val-Ile-Asp-Cys-Gln-Leu-Ala-Asp-Val-Asn-Asn-Ile-Gly-Lys
T 43	430 Ser-Ala-Gly-Ala-Cys-Thr-Ala-Ala-Ala-Phe-Leu-Lys

Fig. 1 The amino acid sequences of the cysteine-containing tryptic peptides of leucine aminopeptidase

L-leucinamide was obtained from Serva; β-mercaptoethanol was from Aldrich; Tris, sodium dodecyl sulfate, and iodoacetic acid were from Sigma; *o*-phenanthroline, $MgCl_2$, $MnCl_2$, guanidine hydrochloride, and urea were from Merck. *N*-methylmorpholine and ethyleneimine were from Pierce. Iodo[2-^{3}H]acetic acid was purchased from the Radiochemical Centre, Amersham (specific activity, 50 mCi/mmol). Sephadex G-25 (coarse) and G-50 (superfine) were from Pharmacia. Trypsin was purchased from Worthington. Picofluor was obtained from Packard. All buffer solutions were prepared with metal-free water, boiled, degassed, and saturated with nitrogen.

Leucine aminopeptidase was prepared as described in the previous paper[11] and dissolved and stored in 0.1 M Tris-HCl buffer, pH 8.0. Purity was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate[12]. Enzyme concentrations were determined by measuring the absorbance at 280 nm, using the molar extinction coefficient of 5.4×10 M^{-1} cm^{-1} for the subunit of 52,000 daltons[1], or by the method of Lowry *et al*[13]. All metal ion exchange reactions, the preparation of apoenzyme and the modification reactions with iodoacetate were performed in an oxygen-free system. All solutions were flushed with nitrogen before use; desalting procedures and modification reactions were performed under a nitrogen barrier. Unbound metal ions and excess of reagents were removed from the enzyme by gel filtration on a Sephadex G-25 (coarse) column, eluted with 0.1 M Tris-HCl buffer, pH 8.6. Also, the native enzyme was desalted on Sephadex G-25 (coarse) to remove unbound Zn^{2+} ions remaining after the isolation procedure.

Preparation of the apoenzyme, Zn^{2+}-Mg^{2+}, Zn^{2+}-Mn^{2+}, and Co^{2+}-Co^{2+} enzyme

The metal-free apoenzyme was prepared by dialysis against *o*-phenanthroline[5] as follows. A volume of 22 ml of Zn^{2+}-Zn^{2+} enzyme (2.4 mg/ml) was dialyzed at 4 °C against 0.25 M Tris-HCl buffer, pH 7.8, containing 0.125 M NaCl, followed by a 10-h dialysis at room temperature against buffer with the same composition but containing in addition 1 mM *o*-phenanthroline, 10 μM β-mercaptoethanol. *o*-Phenanthroline was removed by dialysis at room temperature against 0.25 M Tris-HCl buffer, pH 7.8, containing 0.125 M NaCl, 10 μM β-mercaptoethanol for 8 h.

Zn^{2+}-Mg^{2+} leucine aminopeptidase was prepared by dialysis of Zn^{2+}-Zn^{2+} enzyme (2.5 mg/ml) against 0.1 M Tris-HCl buffer, pH 9.5, containing 1 M KCl,

followed by incubation in the same buffer plus 0.05 M $MgCl_2$, for 12 h at 37 °C[3]. Manganese exchange was performed by dialysis of the Zn^{2+}-Zn^{2+} enzyme (4.5 mg/ml) against 0.02 M Tris-HCl buffer, pH 8.5, containing 1 M KCl and incubation in the same buffer plus $MnCl_2$ at a final concentration of 2 mM for 3 h at 37 °C[1].

Co^{2+}-Co^{2+} leucine aminopeptidase was obtained by dialysis of Zn^{2+}-Zn^{2+} enzyme (4.5 mg/ml) against 0.2 M *N*-methylmorpholine-HCl buffer, pH 7.5, containing 1 M KCl and incubation of this solution during 7 days at 37 °C with $CoCl_2$ at a final concentration of 50 mM[4]. The crystals of the Co^{2+}-Co^{2+} enzyme were collected by centrifugation. In order to dissolve the crystals, a procedure of resuspension in 0.2 M *N*-methylmorpholine-HCl buffer, pH 7.5, and centrifugation (12 times) was used, until most of the crystals were in solution. The final volume of 25 ml, with a protein concentration of 1.9 mg/ml, was made 1 M in KCl and 1 mM in $CoCl_2$. This solution was incubated during 5 h at 37 °C and desalted on a Sephadex G-25 (coarse) column.

Labeling with iodo [2-^{3}H]acetate of the sulfhydryl groups of native Zn^{2+}-Zn^{2+} enzyme, apoenzyme, Zn^{2+}-Mg^{2+}, Zn^{2+}-Mn^{2+}, and Co^{2+}-Co^{2+} enzyme

To determine which sulfhydryl groups react with iodo [2-^{3}H]acetate, the following experiments were performed. To 40 mg of Zn^{2+}-Zn^{2+} enzyme in 30 ml of 0.1 M Tris-HCl buffer, pH 8.6, 79.8 µmol of iodo [2-^{3}H]acetic acid (400 µCi) dissolved in 0.2 ml of 1 M Tris-HCl buffer, pH 8.6, were added dropwise. The pH was maintained at 8.6 with 1 M NaOH. After 15 min, the reaction was stopped by passage of the mixture through a Sephadex G-25 (coarse) column to remove the excess of label. For labeling of apoenzyme and the Zn^{2+}-Mg^{2+} enzyme, the same procedure was used, except that the protein was dissolved in 48 and 54 ml of 0.1 M Tris-HCl buffer, pH 8.6, respectively. In another experiment, 130 µmol of β-mercaptoethanol were added to a solution of 35 mg of apoenzyme in 25 ml of 0.1 M Tris-HCl buffer, pH 8.6. After a reduction time of 1 h, 15 µmol of iodo [2-^{3}H]acetic acid (200 µCi), dissolved in 0.1 ml of 1 M Tris-HCl buffer, pH 8.6, were added and the solution was kept at pH 8.6 for 15 min. Excess reagent was removed by gel filtration. Zn^{2+}-Mn^{2+} leucine aminopeptidase (40 mg) in 80 ml of 0.1 M Tris-HCl buffer, pH 8.6, was treated with 35.6 µmol of iodo [2-^{3}H]acetic acid (400 µCi) in 0.2 ml of 1 M Tris-HCl buffer, pH 8.6, at a constant pH of 8.6 during 15 min. The Co^{2+}-Co^{2+} enzyme (47 mg) was modified in the same way in a volume of 56 ml of Tris buffer. These reactions were also stopped by gel filtration.

Labeling with iodo [2-^{3}H]acetate of the sulfhydryl groups in Zn^{2+}-Zn^{2+} leucine aminopeptidase denatured with 8 M urea

In a typical experiment, solid urea was added, to a final concentration of 8 M, to a solution of 50 mg of enzyme in 10 ml of 0.1 M Tris-HCl buffer, pH 8.0, and the solution was made 20 mM in EDTA. Denaturation was continued during 5 h. The pH was then raised to 8.6 with 4 M NaOH, and 34 µmol of iodo-[2-^{3}H]acetic acid (400 µCi) dissolved in 0.2 ml of 1 M Tris-HCl buffer, pH 8.6, were added dropwise. The pH was kept at 8.6 with 1 M NaOH for 15 min, after which the reaction mixture was passed through a Sephadex G-25 (coarse) column.

In order to ensure a complete blocking of sulfhydryl groups, which had not reacted during the radioactive modification procedures, all protein fractions were alkylated a second time, using unlabeled iodoacetate, by the previously described procedure[11]. Enzymatic digestion, gel filtration of enzymatic digests on Sephadex G-50 (superfine), peptide mapping, and elution of peptides from paper were performed as described[11]. During our sequence studies, we did not find any indication that the alkylated protein was incompletely digested with trypsin. Slight differences found in absorbance profiles of the Sephadex G-50 (superfine) columns as can be seen in Fig. 3, A-F, are not the result of different extents of digestion, but are due to minor differences in the packing of columns and of low molecular weight impurities having absorbance at 280 and 200 nm.

Determination of the incorporated radioactivity was performed by liquid scintillation counting of an aliquot of the fractions collected after gel filtration, or by counting an aliquot of the purified peptides. Samples of up to 0.1 ml in 0.1 M NH_3 were mixed with 2 ml of Picofluor and counted in a Packard Tricarb liquid scintillation counter (Model 3320). Peptides with incorporated ^{3}H label were further characterized by amino acid analysis and determination of the NH_2-terminal residue by the dansyl chloride method[11].

Alkylation with iodoacetate of the Zn^{2+}-Zn^{2+} enzyme and the apoenzyme for the metal readdition experiments was performed in 0.1 M Tris-HCl buffer, pH 8.6, containing 0.01% (v/v) β-mercaptoethanol. To a 4 ml solution of enzyme (2.5 mg/ml) were added 50 mg of iodoacetic acid (neutralized with 4 M NaOH), while the pH was maintained at 8.6. After 10 min, the solution was extensively dialyzed against 0.1 M Tris-HCl buffer, pH 8.0. Readdition of Zn^{2+} ions to the apoenzyme and to the carboxymethylated apoenzyme was performed in 0.25 M Tris-HCl buffer, pH 7.8, containing 0.115 M NaCl, 0.05% (v/v) β-mercaptoeth-

anol. To 8 mg of enzyme in 3 ml of buffer was added 0.15 ml of 0.1 M $ZnCl_2$. The solution was subsequently dialyzed for 2 h at room temperature against 0.01 M Tris-HCl buffer, pH 8.0, containing 0.01 mM $ZnCl_2$, 0.05% (v/v) β-mercaptoethanol, followed by exhaustive dialysis against 0.1 M Tris-HCl buffer, pH 8.0.

Enzymatic activity measurements were carried out as described[14] with two modifications: activation of the enzyme was omitted and no $MgCl_2$ was added to the incubation mixture. The protein concentration in the incubation mixture was approximately 0.05 mg/ml. Activity was expressed as micromoles of L-leucinamide hydrolyzed/min/mg of enzyme.

All seven tryptic peptides containing [^{3}H]carboxymethylcysteine could easily be purified by gel filtration, followed by peptide mapping of the pooled fractions[11]. A tryptic digest of denaturated, reduced, and [2-^{3}H]iodoacetate-alkylated leucine aminopeptidase was applied to a Sephadex G-50 (superfine) column (see Fig. 17 in Ref. 11). Pool I yielded peptide T 14 in pure form. Pools II to VI were further purified by peptide mapping (Fig. 2). The peptides were found to be homogeneous on the basis of amino acid analyses and determination of the NH_2-terminal residues. The amino acid compositions are presented in Table XX of Ref. 11. The same method of analysis was used in all

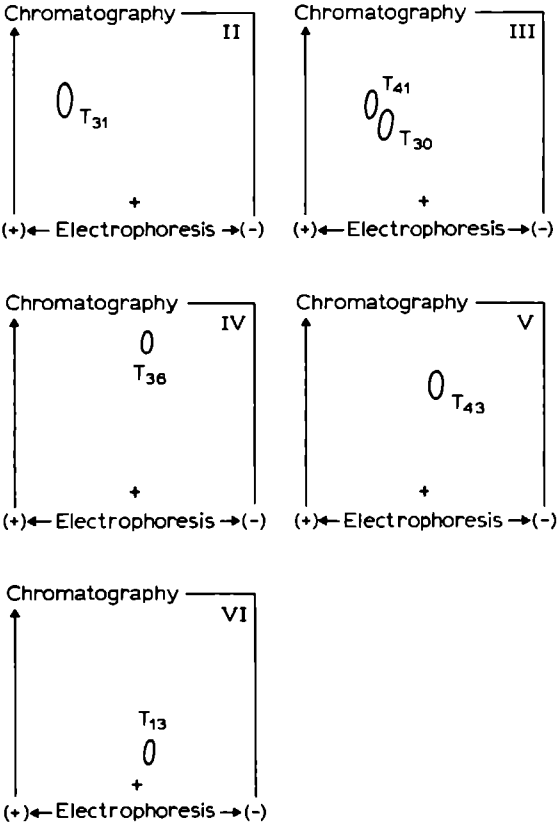

Fig. 2 Peptide mapping of the tryptic peptides of denatured, reduced, and [2-^{3}H]-iodoacetate-alkylated leucine aminopeptidase. Pool fractions of the Sephadex G-50 (superfine) column (see Fig. 17 in Ref. 11) were applied at the origin (+) and subjected to high voltage electrophoresis at pH 6.5 followed by descending chromatography in the second dimension. Peptides were visualized with fluorescamine and an aliquot of the eluted peptides was subjected to liquid scintillation counting. The peptides containing [^{3}H]cysteine are marked.

labeling experiments. Because the modification experiments were performed with different concentrations of iodoacetate in the reaction mixture, specific activities of the individual cysteine residues (Table I) are only comparable

Table I *Incorporation of ^3H label in differently modified forms of leucine aminopeptidase by alkylation with iodo [2-^3H]acetate*

For experimental details, see 'Materials and Methods'. The specific activities of the different cysteine residues were determined on the basis of liquid scintillation counting and amino acid analysis and is expressed as counts/min/nmol

Cysteine residue	Type of modifications*							
	A	B	C	D	E	F	G	H
97	0	0	0	0	0	40	1897	2231
113	0	0	0	0	0	43	1320	1826
281	0	0	0	24	21	234	1444	2167
303	0	23	0	42	200	464	1395	2040
344	1615	545	722	135	563	699	1843	2335
412	122	409	0	832	1097	1502	1104	1935
429	115	71	0	20	357	860	2130	2409

*A, native leucine aminopeptidase (= Zn^{2+}-Zn^{2+}); B, Zn^{2+}-Mg^{2+} enzyme; C, Zn^{2+}-Mn^{2+} enzyme; D, Co^{2+}-Co^{2+} enzyme; E, metal-free apoenzyme; F, metal-free apoenzyme treated with β-mercaptoethanol; G, enzyme denatured with 8 M urea and reduced with β-mercaptoethanol; H, enzyme denatured with 8 M urea.

within the same labeling experiment. All the labeling experiments were performed twice, but with different concentrations of iodoacetate. The relative distribution of label over the different cysteine residues was identical in both cases. The experiments giving the highest specific activities are presented in Table I.

Determination of the reactivity of the sulfhydryl groups in denaturated Zn^{2+}-Zn^{2+} leucine aminopeptidase

Upon determination of the reactivity of the sulfhydryl groups of the enzyme denatured with 8 M urea, the elution pattern of labeled tryptic peptides from the gel filtration column was identical with that found after denaturation, reduction, and alkylation of the enzyme (see Fig. 17 of Ref. 11). The specific activity of [^3H]carboxymethylcysteine in the different peptides was determined (Table I). It was found that in the native enzyme all cysteines are in the sulfhydryl form. The same results were obtained when 4.8 M guanidine hydrochloride was used as denaturing agent (results not shown).

Determination of the reactivity of the sulfhydryl groups in Zn^{2+}-Zn^{2+}, Zn^{2+}-Mg^{2+}, Zn^{2+}-Mn^{2+}, and Co^{2+}-Co^{2+} leucine aminopeptidase

Fig. 3A shows the elution pattern of the Sephadex G-50 (superfine) column of the tryptic peptides of native Zn^{2+}-Zn^{2+} enzyme, modified with iodo [2-^{3}H]-acetate. The two pooled fractions, containing ^{3}H label, were analyzed by means of peptide mapping (Table I). This revealed a high incorporation in peptide T 36, indicating a high reactivity of cysteine-344 toward iodoacetate. Two other cysteines, residue 412 (peptide T 41) and residue 429 (peptide T 43), showed a much lower reactivity, approximately 7% of the labeling of cysteine 344.

The gel filtration pattern of the tryptic digest of the Zn^{2+}-Mg^{2+} enzyme, modified with iodo [2-^{3}H] acetate is shown in Fig. 3B. Analysis of the radioactive fractions by peptide mapping (Table I) revealed a high incorporation into two cysteines: residue 344 (peptide T 36) and residue 412 (peptide T 41), and a low incorporation (13% of the labeling of cysteine-344) into cysteine-429 (peptide T 43). The results of tryptic digestion of the Zn^{2+}-Mn^{2+} enzyme labeled with iodo [2-^{3}H] acetate are given in Fig. 3C and Table I. Only one cysteine, residue 344 (peptide T 36), has a high reactivity toward iodoacetate.

Because the procedure of resuspension of crystals of the Co^{2+}-Co^{2+} enzyme might result in the loss of cobalt ions from the enzyme, the solution was preincubated with 1 mM $CoCl_2$ in a buffer containing 1 M KCl[4]. After modification of the Co^{2+}-Co^{2+} enzyme with iodo [2-^{3}H] acetate, the tryptic digest was chromatographed on G-50 (superfine) (Fig. 3D), followed by peptide mapping. The detected incorporation is presented in Table I. Cysteine at position 412 (peptide T 41) is the most reactive, followed by cysteine-344 (peptide T36) which has a reactivity of 16% of the value determined for cysteine-412.

Determination of the reactivity of the sulfhydryl groups in the metal-free apoenzyme

The reactivity of the sulfhydryl groups in the metal-free apoenzyme was determined in a system without reducing agent and in a system to which β-mercaptoethanol was added. Fig. 3E shows the gel filtration pattern of a tryptic digest of the apoenzyme modified with iodo [2-^{3}H] acetate in the absence of reducing agent. Peptide mapping of the pooled fractions revealed four cysteine residues susceptible to modification by iodoacetate (Table I). Cysteine-412 (peptide T 41) is the most reactive, followed by cysteine-344 (peptide T 36), cysteine-429 (peptide T 43), and cysteine-303 (peptide T 31), with incorporation values of 51, 33 and 18%, respectively, as compared to the specific ac-

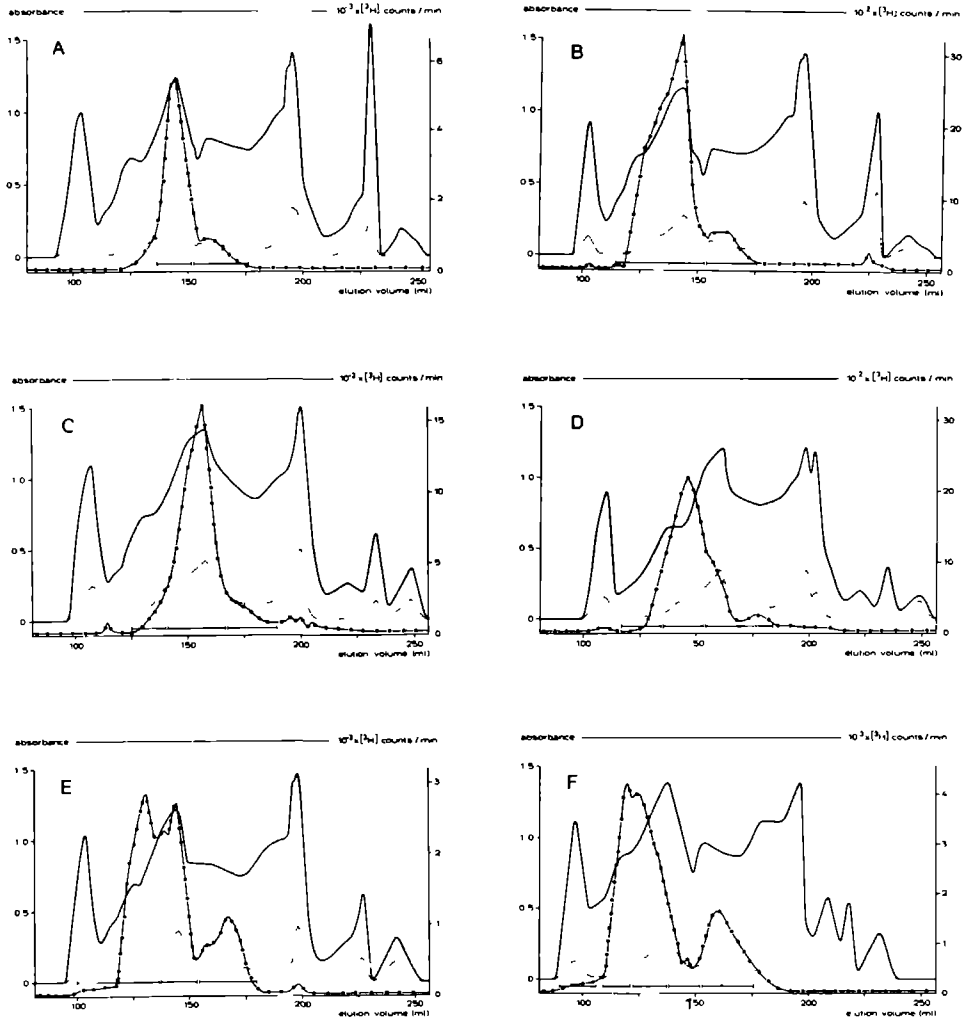

Fig. 3 Gel filtration of tryptic peptides obtained from native and modified forms of leucine aminopeptidase labeled with iodo[2-^{3}H]acetate. Labeling was performed as described under 'Materials and Methods'. The tryptic digest of the labeled enzyme was applied to a Sephadex G-50 (superfine) column (120 x 1.6 cm) in 0.1 M ammonia and eluted with the same solvent at a flow rate of 6.6 ml/h. Absorbance was measured at 206 (——) and 280 nm (---). Fractions of 2.2 ml were collected, of which an aliquot was used for radioactivity measurement (●——●). Fractions were pooled as indicated and further purified by peptide mapping. A, native Zn^{2+}-Zn^{2+} enzyme. An aliquot of 70 µl of the fractions was counted. B, Zn^{2+}-Mg^{2+} enzyme; 150 µl were counted. C, Zn^{2+}-Mn^{2+} enzyme; 150 µl were counted. D, Co^{2+}-Co^{2+} enzyme; 100 µl were counted. E, metal-free apoenzyme; 70 µl were counted. F, metal-free apoenzyme labeled in the presence of β-mercaptoethanol; 100 µl were counted.

tivity of cysteine-412. In presence of 5.2 mM β-mercaptoethanol the same method of analysis gives the gel filtration pattern shown in Fig. 3F. In Table I, the specific activities of the individual cysteine residues are listed. Once more, cysteine-412 (peptide T 41) was the most reactive residue. Cysteine residues 429, 344, 303, and 281 had specific activities amounting to 57, 47, 31, and 16%, respectively, of the value determined for position 412.

Activity measurements on carboxymethylated Zn^{2+}-Zn^{2+} enzyme and on carboxymethylated apoenzyme incubated with Zn^{2+} ions

Blocking of the sulfhydryl groups in native Zn^{2+}-Zn^{2+} leucine aminopeptidase resulted in an enzyme with roughly the same activity as the native enzyme (Table II). The activity of the metal-free apoenzyme can be restored to a level of 56% of the activity of the starting material by addition of Zn^{2+} ions (Table II). However, when the sulfhydryl groups of the apoenzyme are first blocked with iodoacetate, the enzyme activity cannot be restored by Zn^{2+} ions (Table II).

Table II Activity measurements on native and modified leucine aminopeptidase

Modifications involved carboxymethylation of Zn^{2+}-Zn^{2+} enzyme, removal and readdition of zinc to the native enzyme, and readdition of zinc to the carboxymethylated apoenzyme. Carboxymethylation was performed as described under 'Material and Methods'. Activity was measured using 0.072 to 0.120 mg of enzyme in 2.4 ml of 0.02 M Tris-HCl buffer, pH 8.5, with 0.125 mmol of L-leucinamide

	specific activity	activity
	μmol/min/mg	%
Starting material	70.9 ± 3.5	100
Carboxymethylated zinc-zinc enzyme	66.0 ± 3.0	93
Metal-free apoenzyme	0	0
Zinc-zinc enzyme reconstituted from apoenzyme	39.8 ± 3.0	56
Readdition of zinc ions to carboxymethylated apoenzyme	0	0

Our results indicate that in native leucine aminopeptidase all seven cysteines in each subunit are in the sulfhydryl form. The elucidation of the primary structure revealed the presence of seven half-cystines and the determination of the specific activities of the enzyme after denaturation, reduction, and alkylation showed that all cysteines are in the sulfhydryl form. A value of 6.3 sulfhydryl groups/subunit found by the reaction of the enzyme with iodoacetamide after extensive denaturation[1] might have been caused by incomplete blocking of the sulfhydryl groups. Since other authors[1,6] could not discriminate among the reactivities of the individual sulfhydryl groups, the conclusion that one disulfide bridge is present in the native enzyme may be doubted.

In the Zn^{2+}-Zn^{2+} enzyme, one cysteine (residue 344) has a high reactivity toward iodoacetate, and two cysteines (residue 412 and 429) have a reactivity of only approximately 7% of that determined for cysteine 344 (Table I). From our results in combination with those of Carpenter and Vahl[1], we conclude that cysteine-344 in the native enzyme was almost completely modified by iodoacetate. The value of 1.18 sulfhydryl groups/subunit[1] may result from the complete blocking of cysteine-344 and partial (less than 10%) blocking of cysteines 412 and 429. In the case of the Zn^{2+}-Mn^{2+} enzyme, approximately the same results were obtained for the reactivity of the cysteine residues toward iodoacetate. Only cysteine-344 was found to be reactive.

In the Zn^{2+}-Mg^{2+} and the Co^{2+}-Co^{2+} enzyme, other cysteine residues become reactive toward iodoacetate. Frohne and Hanson[10] determined a value of 6.7 reactive sulfhydryl groups/molecule at pH 8.6 in the Zn^{2+}-Zn^{2+} enzyme, and 9.4 reactive sulfhydryl groups in the Zn^{2+}-Mg^{2+} enzyme. The increased reactivity of cysteine 412 could explain this higher value in the Zn^{2+}-Mg^{2+} enzyme. In the Co^{2+}-Co^{2+} enzyme, cysteine-412 was the most reactive sulfhydryl group, and cysteine-344 showed only 16% of the reactivity determined for residue 412.

In the metal-free apoenzyme, even more sulfhydryl groups become reactive. Frohne and Kettmann[6] found a value of 24/molecule in the apoenzyme. From Table I, it may be concluded that this reactivity can be attributed to cysteine residues 412, 344, 429, and 303, in order of decreasing reactivity. When the apoenzyme was treated with β-mercaptoethanol, a procedure used for reactivation of the apoenzyme with Zn^{2+} ions, there was an additional enhance-

ment of the reactivity of cysteine residues 281, 303, and 429. In all labeling experiments with native leucine aminopeptidase, cysteine residues 97 and 113 remained completely unreactive toward iodoacetate.

Blocking of cysteine-344 with iodoacetate in Zn^{2+}-Zn^{2+} leucine aminopeptidase resulted only in a minor change of the enzyme activity. This thiol group is therefore not directly involved in enzymic activity. Hence, native leucine aminopeptidase cannot be classified as a 'classical' sulfhydryl enzyme, in which reactive sulfhydryl groups are closely linked with enzymatic activity. It is possible, however, that cysteine-344 is involved in an essential intermediate step of the activation[9]. It should be realized that chemical modification of proteins, involving change of charge at reactive groups, sometimes leads to dissociation or aggregation, which in turn can affect the enzyme activity and can bring about further modification reactions[16]. Modifications with iodoacetamide might be helpful in evaluation of the role of charge in the alkylating group. However, in the case of leucine aminopeptidase, it is very difficult to separate the labeled peptides when [^{3}H]iodoacetamide is used as alkylating agent.

Among the Zn^{2+} metalloenzymes, for which the tertiary structures have been determined (carbonic anhydrase, carboxypeptidase, thermolysin, superoxide dismutase and alcohol dehydrogenase), only alcohol dehydrogenase has zinc ions liganded to cysteinyl sulfur. Horse liver alcohol dehydrogenase contains two firmly bound zinc ions: a catalytic zinc ion, which is liganded to two sulfur atoms in cysteine residues, and a second zinc ion liganded to sulfur atoms from four cysteines[17]. In leucine aminopeptidase, the Zn^{2+} ions may also be liganded to cysteinyl sulfur. It is possible that cysteine-412 becomes reactive toward iodoacetate in the Zn^{2+}-Mg^{2+} enzyme as a result of the occupation of the activation site by magnesium, which makes the coordination of cysteine-412 and Zn^{2+} impossible. It is known that Mg^{2+} binds so weakly to sulfhydryl groups that they do not form chelation complexes[18]. Another explanation could be that activation with magnesium ions brings about a change of conformation, resulting in the exposure of cysteine-412. On the other hand, Mn^{2+} does form complexes with cysteine[18], which might explain that in the Zn^{2+}-Mn^{2+} enzyme cysteine-412 does not react with iodoacetate.

The involvement of sulfhydryl groups in the liganding of Zn^{2+}-ions in leucine aminopeptidase is also supported by the following observation. Blocking with iodoacetate of the sulfhydryl groups in the apoenzyme, followed by incubation with Zn^{2+} ions, does not restore the enzymatic activity. A possible

explanation is that Zn^{2+} can no longer be bound to the blocked enzyme. If Zn^{2+} forms chelation complexes with cysteines, it is likely that cysteine-412 is involved in this ligand formation, but coordination with other cysteines is also possible. Elucidation of the tertiary structure of leucine aminopeptidase will provide the final answer to these questions.

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The enzyme leucine aminopeptidase catalyzes the *N*-terminal release of amino acid residues from peptides and proteins. The enzyme has its highest activity toward hydrophobic residues. Leucine aminopeptidase has a molecular weight of 326,000 and consists of six identical subunits with an apparent molecular weight of 54,000. It belongs to the class of aminopeptidases, which are widely distributed in plants, microorganisms and animal tissues. No primary structure of a member of this class of enzymes had hitherto been published. We, therefore, have elucidated, in collaboration with Dr L. van Loon-Klaassen, the primary structure of leucine aminopeptidase from bovine eye lens.

A short literature survey of the data on leucine aminopeptidase from bovine lens and hog kidney is presented in Chapter I. The properties of some other mammalian aminopeptidases, especially those which are membrane-bound, are also described. In connection with some recent views on intracellular breakdown of proteins in mammalian cells, the possible role of leucine aminopeptidase in this system is discussed. Finally, data concerning the proteolysis in bovine lens are surveyed, and a speculation is made about the functions of aminopeptidases in the lens, especially for leucine aminopeptidase, which represents the major part of the activity and is present in abundance.

Chapter II describes the strategy and the results of the sequence analysis of the subunit of leucine aminopeptidase. The complete primary structure has been established by:

1. cleavage with cyanogen bromide at the methionine residues, yielding 13 fragments, of which 11 could partially or completely be purified and sequenced
2. splitting of the only Asn-Gly bond by hydroxylamine and purification of the *C*-terminal fragment and subsequent determination of the sequence of this part of the polypeptide chain
3. purification and sequence ascertainment of a large fragment obtained by digestion with *Staphylococcus aureus* protease
4. tryptic digestion of the *S*-alkylated polypeptide chain or the citraconylated *S*-alkylated chain yielded overlapping peptides. The sequence of these peptides was determined and allowed the arrangement of all fragments in the correct order.

The proposed amino acid sequence of bovine lens leucine aminopeptidase is presented in Fig. 4, page 32-33. The polypeptide chain of leucine aminopeptidase

comprises 478 residues, corresponding to a molecular weight of 51,691. These results represent the first complete primary structure determination of a member of the class of aminopeptidases. No significant sequence homology with any other published protein primary structure could be detected, which places leucine aminopeptidase in a separate protein superfamily.

In Chapter III the exposure of the sulfhydryl groups of leucine aminopeptidase, as determined by the reactivity toward iodoacetate, is described for the Zn^{2+} metalloenzyme and the enzyme activated by Mg^{2+}, Mn^{2+}, and Co^{2+}. The reactivity of the sulfhydryl groups has also been determined in the presence of denaturing agents, with or without reducing conditions. It appeared that all seven half-cystines per leucine aminopeptidase subunit are in the sulfhydryl form. In the native (Zn^{2+}-Zn^{2+}) and the Zn^{2+}-Mn^{2+} enzyme, only one of the cysteines (residue 344) reacts readily with iodoacetate. In the Zn^{2+}-Mg^{2+} enzyme two cysteines react (residues 344 and 412), and in the Co^{2+}-incubated enzyme (Co^{2+}-Co^{2+}) only one (residue 412). The metal-free apoenzyme has at least three reactive cysteines (residues 344, 412 and 429). While incubation of the unblocked apoenzyme with Zn^{2+} ions results in restoration of enzymatic activity, no such recovery of enzymatic activity is obtained after blocking with iodoacetate of the sulfhydryl groups in the apoenzyme. We conclude from these results that if Zn^{2+} forms chelation complexes with cysteines, it is likely that cysteine-412 is involved in the ligand formation, but coordination with other cysteines is also possible.

Het enzym leucine aminopeptidase katalyseert de afsplitsing van N-eindstandige aminozuren van peptiden en eiwitten. Hydrofobe aminozuren worden met de grootste snelheid afgesplitst. Leucine aminopeptidase heeft een molecuulgewicht van 326.000 en is samengesteld uit zes gelijkvormige subeenheden met een molecuulgewicht van 54.000. De aminopeptidasen spelen een rol bij de intracellulaire afbraak van eiwitten. Tot nu toe was van geen van de aminopeptidasen een aminozuurvolgorde bepaald. Ons onderzoek was gericht op de opheldering van de aminozuurvolgorde van runderooglens leucine aminopeptidase. Dit onderzoek is uitgevoerd in samenwerking met Dr L. van Loon-Klaassen.

De literatuurgegevens over leucine aminopeptidase uit de runderooglens en het nauw verwante enzym uit het cytosol van de varkensnier zijn samengevat in Hoofdstuk I. Ook worden de eigenschappen beschreven van enkele andere zoogdier-aminopeptidasen die membraangebonden zijn. De functies die leucine aminopeptidase mogelijkerwijs heeft bij de afbraak van eiwitten binnen zoogdiercellen worden besproken in samenhang met recente inzichten over deze mechanismen. Tot slot worden de gegevens over de eiwitafbraak in de ooglens samengevat en wordt bekeken, welke functies aminopeptidasen hierbij zouden kunnen hebben - in het bijzonder het leucine aminopeptidase - dat in grote hoeveelheid voorkomt en de hoogste aktiviteit heeft.

De methoden van onderzoek en de resultaten van de aminozuurvolgordebepaling van de subeenheid van leucine aminopeptidase worden beschreven in Hoofdstuk II. De bepaling van de volledige volgorde kan als volgt worden samengevat:

1. de polypeptideketen is met cyanogeenbromide gesplitst naast de methionines wat 13 fragmenten oplevert. Elf cyanogeenbromidefragmenten zijn gedeeltelijk of volledig gezuiverd en de aminozuurvolgordes van deze fragmenten zijn bepaald
2. de enige Asn-Gly-binding is verbroken met behulp van hydroxylamine en het C-eindstandige fragment dat hieruit resulteert, is opgezuiverd en de volgorde ervan bepaald
3. een groot fragment van de polypeptideketen is verkregen na digestie met *Staphylococcus aureus* protease. De volgorde van dit fragment is vastgesteld
4. behandeling met trypsine van de S-gealkyleerde polypeptideketen, zowel met als zonder blokkering van lysineresiduen door citraconylering, levert pep-

tiden op die de overlap tussen de verkregen deelvolgordes op de juiste wijze vastlegt. De volgordes van deze peptiden zijn bepaald.

De voorgestelde aminozuurvolgorde van het runderooglens-leucine aminopeptidase is in Fig. 4, pag. 33, weergegeven. De volledige polypeptideketen van leucine aminopeptidase omvat 478 residuen, wat overeenkomt met een molecuulgewicht van 51.691. Dit is de eerste volledige aminozuurvolgorde-bepaling van een enzym uit de klasse van de aminopeptidasen. De volgorde vertoont geen homologie van enige betekenis met andere gepubliceerde eiwitvolgordes. Leucine aminopeptidase moet op grond hiervan in een aparte eiwit-superfamilie geplaatst worden.

In Hoofdstuk III wordt de bereikbaarheid van de sulfhydrylgroepen van leucine aminopeptidase beschreven in het zink-metaalenzym en in het enzym, geaktiveerd met Mg^{2+}, Mn^{2+} en Co^{2+}. Hiertoe werd de reaktiviteit ten opzichte van joodacetaat bepaald. De reaktiviteit van de sulfhydrylgroepen is ook in aanwezigheid van denaturerende reagentia bepaald, met en zonder een reducerend agens. Geconcludeerd kan worden dat alle zeven half-cystines die voorkomen in de leucine aminopeptidase-subeenheid, in de sulfhydrylvorm zijn. In het natieve (Zn^{2+}-Zn^{2+}) en het Zn^{2+}-Mn^{2+} enzym reageert slechts één van de cysteïnes (positie 344) met joodacetaat. In het Zn^{2+}-Mg^{2+} enzym reageren twee cysteïnes (posities 344 en 412) en in het met Co^{2+} geïncubeerde enzym (Co^{2+}-Co^{2+}) slechts één (positie 412). Het apoenzym dat geen metaal-ion bevat, heeft ten minste drie reaktieve cysteïnes (posities 344, 412 en 429). Wanneer de sulfhydrylgroepen in het apoenzym met joodacetaat geblokkeerd worden, en vervolgens het geblokkeerde apoenzym met Zn^{2+} geïncubeerd wordt, vindt geen herstel van de enzym-aktiviteit plaats. Een dergelijk herstel vindt wél plaats door incubatie met Zn^{2+} van het niet gemodificeerde apoenzym. Uit deze resultaten kan geconcludeerd worden dat cysteïne-412 waarschijnlijk betrokken is bij de vorming van Zn^{2+} chelaat complexen, maar coördinatie met andere cysteïnes is ook mogelijk.

H. Theo M. Cuypers werd geboren op 19 september 1949 te Eindhoven.

Na het behalen van het eindexamen HBS-B in 1967 aan het "Pax-Christi College te Druten begon hij de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S1) werd in 1970 afgelegd. De doctoraalstudie omvatte de hoofdrichting Organische Chemie en de bijvakken Moleculaire Biologie en Biochemie.

Gedurende het schooljaar 1971/1972 was hij leraar scheikunde aan het "College van het Heilige Kruis" te Uden. Vanaf maart 1975 t/m mei 1976 vervulde hij de militaire dienstplicht. In mei 1976 werd het doctoraalexamen behaald.

Vanaf juni 1976 t/m juli 1980 was hij als wetenschappelijk medewerker verbonden aan de Afdeling Biochemie, waar - onder leiding van dr W.W. de Jong en Prof.dr H. Bloemendal - het in dit proefschrift beschreven onderzoek werd verricht.

Vanaf juli 1980 t/m november 1982 was hij in dienst van de Stichting voor Medisch Wetenschappelijk Onderzoek Fungo als wetenschappelijk medewerker verbonden aan de Afdeling Interne Geneeskunde van het St. Radboudziekenhuis.

Sinds december 1982 is hij weer werkzaam op de Afdeling Biochemie om onderzoek te verrichten aan virus geïnduceerde leukemogenese bij muizen.

I

Leucine aminopeptidase kan op basis van de bepaalde aminozuurvolgorde in een aparte eiwit-superfamilie geplaatst worden.

Dit proefschrift, Hoofdstuk II.

II

De veronderstelling dat de eiwitsamenstelling in de verschillende lenslagen van enige vissoorten het verloop van de fylogenetische ontwikkeling zou weerspiegelen, analoog aan de door Haeckel geformuleerde biogenetische grondwet, wordt niet ondersteund door de verkregen resultaten.

Smith, A.C. (1982) Comp. Biochem. Physiol. 71B, 723-726.

III

De door Khan *et al* in de titel aangekondigde identificatie van een envelop-voorouder van AKR MCF-247 proviraal DNA blijkt bij lezing van het artikel niet bewezen te worden.

Khan, A.S., Rowe, W.P. en Martin, M.A. (1982) Journal of Virology 44, 625-636.

IV

De bewering van De Klein *et al*, dat de ontrafeling van de organisatie van humane genetische sequenties met homologie voor het virale oncogen v-*abl* leidt tot de identificatie van het gehele c-*abl* gen bij de mens is onjuist en getuigt van wetenschappelijke onzorgvuldigheid van enkele van haar medeauteurs.

De Klein, A., Geurts van Kessel, A., Grosveld, G., Bartram, C.R., Hagemeyer, A., Bootsma, D., Spurr, N.K., Heisterkamp, N., Groffen, J. en Stephenson, J.R., Nature 300, 765-767.
Heisterkamp, N., Groffen, J. en Stephenson, J.R. (1983) Journal of Molecular and Applied Genetics 2, 57-68.

V

Lage concentraties bilirubine en bilirubine-conjugaten (<60 µmoles) in het serum van patiënten kunnen nauwkeurig bepaald worden met behulp van hoge druk vloeistofchromatografie.
Bepaling door middel van de diazomethode, veelal toegepast op klinisch chemische laboratoria, geeft onbetrouwbare resultaten in het lage concentratiegebied.

Scharschmidt, B.F., Blanckaert, M., Farina, F.A., Kabra, P.M., Stafford, B.E. en Weisiger, R.A. (1982) Gut 23, 643-649.

VI

De gedeeltelijke deficiëntie van UDP-glucuronyltransferase in de lever van patiënten met het Gilbert-syndroom en in Gunn-ratten berust waarschijnlijk op een verschillend mechanisme.

Nakata, D., Zakim, D. en Vessey, D.A. (1976) Proc. Natl. Acad. Sci. 73, 289-292.
Peters, W.H.M., persoonlijke mededeling.

VII

Bilirubine-diglucuronide vorming in rattelevermicrosomen kan als een twee-stapsreactie beschreven worden.

Cuypers, H.T.M., Ter Haar, E.M. en Jansen, P.L.M. (1983) Biochem. Biophys. Acta 758, 135-143.

VIII

De eigenschap van het humane T-cel leukemie virus (HTLV) om met name T-cellen te infecteren, kan verklaard worden door de verwantschap tussen het virale envelop-eiwit en klasse I histocompatibiliteits-antigenen.

Clarke, M.F., Gelmann, E.P. en Reitz, M.S. (1983) Nature 305, 60-62.
Gallo, R.C., Mann, D., Broder, S., Ruscetti, F.W., Maeda, M., Kalyanaraman, V.S., Robert-Guroff, M. en Reitz, M.S. (1982) Proc. Natl. Acad. Sci. 79, 5680-5683.

IX

De nadruk die op dit moment gelegd wordt op de beoordeling van het wetenschappelijk onderzoek van de vakgroepen op universiteiten en hogescholen en de daaruit voortkomende methode van financiering van het onderzoek, kan in de toekomst leiden tot een verschraling van het onderwijs.

X

Het instellen van een "verplicht" uurtje rikken in de middagpauze in plaats van een "discussie"uurtje draagt bij tot een prettige werksfeer.

H.T.M. Cuypers

Nijmegen, 17 november 1983